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This report documents the continued research and development of BoNTC Hc, BoNTE Hc and BoNTF Hc. A new approach to fermentation of BoNTC Hc was developed that incorporated a mixed feed of glycerol and methanol during the induction. It was determined that the optimum growth rate for expression of BoNTC Hc during methanol induction was 0.015 h^{-1} and the range was very limited. The growth rate range was extended to 0.025 h^{-1} by supplementing the methanol feed rate with a feed of glycerol. BoNTE Hc research focused only on purification development and a four step process produced over 98% pure BoNTE Hc based on SDS-PAGE. Two steps that were critical to the purification process, a batch capture step using anion exchange as BoNTE Hc would not completely bind (40 to 50% in column flow through) under dynamic conditions. A batch process was able to capture nearly 95% of BoNTE Hc. The last step was, hydrophobic interaction chromatography removed a 17 kD *Pichia pasrtoris* protein that was also problematic with BoNTA Hc. Key to the HIC step was addition of 5% glycerol to the buffer system. BoNTF Hc research focused on optimizing a process transferred to UN-L from USAMRIID through Covance.

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FOREWORD

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Introduction

The BPDF has been working on developing processes to produce BoNT A, B, C, E and F over the past six years. This final report is for the project year of 2001. This report is structured to discuss the results of work completed over the last year for work that has been performed on serotypes C, E and F. For the readers reference is a list of manuscripts that have resulted on work conducted on serotypes A and B. As mentioned above, this report describes work on the ferementation and purification development of BoNTC, E and F Hc expressed in *Pichia pastoris.*. In some cases, a section of the report is a manuscript that has either been submitted or is in preparation. Eventually all of the work described in this report will be published. In several cases BoNTC and E, there is additional work at UN-L that is necessary to complete a manuscript. In the case of BoNTF Hc, we are awaiting

Body

1. BoNTA Hc

1. Potter, K.J., W. Zhang, L.A. Smith, and M.M. Meagher, 2000. Production and purification of the Heavy Chain Fragment C of Botulinum Neurotoxin, Serotype A, expressed in the methylotropic yeast *Pichia pastoris*. Protein Express. Purif. **19**:393-402.
2. W. Zhang, M. Inan and M. M. Meagher (2000). Fermentation strategies for recombinant protein expression in the methylotrophic yeast *Pichia pastoris*. Biotechnol. Bioprocess Eng. **5** (4): 275-287.
3. Zhang, W., M.A. Bevins, B.A. Plantz, L.A. Smith, and M.M. Meagher, 2000. Modeling *Pichia pastoris* Growth on Methanol and Optimizing the Production of a Recombinant Protein, the Heavy-Chain Fragment C of Botulinum Neurotoxin, Serotype A. Biotechnol. Bioeng. **70** (1): 1-8.

2. BoNTB Hc

1. Potter, K. J., M. A. Bevins, E. V. Vassilieva, V. J. Chiruvolu, T. Smith, L. A. Smith, M. M. Meagher, 1998. Production and purification of the heavy-chain fragment C of botulinum neurotoxin, serotype B, expressed in the methylotropic yeast *Pichia pastoris*. Protein Express. Purif. **13**: 357-365.

3. BoNTC Hc

Fermentation

Below is the entire manuscript that was submitted on the BoNTC Hc fermentation process. The manuscript provides a complete summary of the fermentation research complete during this time period.

***Pichia pastoris* Fermentation with Mixed-Feeds of Glycerol and Methanol: Growth Kinetics and Production Improvement**

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Sponsor: United States Army Medical Research and Materiel Command (Contract number: DAMD-17-98-C-8034)

ABSTRACT

Fed-batch fermentation of methanol utilization plus (Mut^+) strains of *Pichia pastoris* typically use glycerol as the carbon source for cells in growth phase and methanol as both inducer and carbon source for expression of recombinant proteins during production phase. The literature has reported mixed feeding with glycerol and methanol simultaneously during induction (production) phase to attempt to improve the fermentation. However, growth kinetics on glycerol and methanol and the interaction between them were not reported. The objective of this paper is to optimize the mixed feeding strategy based on the growth kinetic studies using a Mut^+ *Pichia* strain as a model system, which intracellularly expresses the heavy-chain fragment C of botulinum neurotoxin serotype C [BoNT/C(Hc)]. Growth models on glycerol and methanol that describe the relationship between specific growth rate (μ) and specific glycerol/methanol consumption rate (v_{gly}, v_{MeOH}) were established. Mixed feeding strategy with desired μ_{gly}/μ_{MeOH} = 1, 2, 3, 4 (desired μ_{MeOH} set at 0.015 h^{-1}) were employed to study the growth interactions and its effect on production. The results show the optimal desired μ_{gly}/μ_{MeOH} is around 2 for obtaining the highest BoNT/C(Hc) protein content in cells, which is about 3 mg/g wet cells. The optimal feed rate ratio of glycerol:methanol in the mixed feed was derived to be 0.889, based on desired μ_{gly}/μ_{MeOH} = 2; and the actual overall growth rate obtained under this feeding strategy was 0.5 h^{-1}. The method developed in this paper can be applied to mixed feed designs for both Mut^+ and Mut^s *Pichia* fermentations producing various recombinant proteins.

Keywords: *Pichia pastoris*; fed-batch fermentation; mixed feed; optimization; growth model; botulinum neurotoxin.

INTRODUCTION

In the genome of the methylotrophic yeast *Pichia pastoris*, there are two copies of the alcohol oxidase (AOX) gene, designated *AOX1* and *AOX2*. These genes enable the cells to assimilate methanol as the sole carbon and energy source. The *AOX1* promoter regulates 85% of the alcohol oxidase production while the *AOX2* promoter is less active (Cregg et al., 1989). The *Pichia* expression system uses the *AOX1* promoter to drive heterologous protein expression with methanol as the inducer. Through gene disruption (Cregg and Madden, 1987), the "*AOX1* promoter-interesting gene" expression cassette is inserted in the genome (Cregg et al., 2000; Cregg et al., 1989; Romanos et al., 1992). Depending on the locus of insertion, two different phenotypes of *Pichia* are generated: methanol utilization plus (Mut^+) or methanol utilization slow (Mut^s). The former contains both *AOX1* and *AOX2*, while the later only *AOX2*. For Mut^s strains, due to its slow utilization of methanol, a mixed feed of glycerol and methanol is commonly employed in the fermentation induction phase. Here glycerol functions as an efficient substrate for cell growth and target protein production while methanol functions as an inducer. With this strategy, various proteins were successfully expressed in either fed-batch or continuous operation mode by Mut^s strains (Brierley et al., 1990; d'Anjou and Daugulis, 2001; Loewen et al., 1997; Sreekrishna et al., 1989). Similarly, Chiruvolu et al. (1997) compared the intracellular expression of single-gene copy constructs β-galactosidase of both a Mut^+ and Mut^- strain. Chiruvolu et al. determined that Mut^-, which is unable to metabolize methanol produced nearly 50% more enzyme using a glycerol feed rate of 1 g/L/h at a methanol concentration 0.5%. The Mut^- fermentation followed a linear increase in methanol during induction to a final MeOH feed rate of 9 ml/l/h. Chiruvulo et al. found that as the glycerol feed rate increased from 1 to 7 g/L/h the amount of enzyme per unit cell mass decreased. In all of these studies the optimization of mixed feeding strategy was based on arbitrary ratios of the two substrates in the feed solution. Growth kinetics on glycerol and methanol, and the interaction between them were not studied.

For Mut^+ strains of *Pichia*, a typical fed-batch fermentation strategy is to feed methanol alone as both inducer and carbon and energy source in the induction phase due to its efficient utilization of methanol. We recently developed an exponential feeding strategy that is based on a growth model describing the relationship between specific growth rate and specific methanol consumption rate (Zhang et al., 2000). This makes it possible to maximize production based on an optimal specific growth rate. A basic protocol book, "*Pichia* Fermentation Process Guidelines" is also available from Invitrogen Co. (San Diego, CA), which provides a methanol feeding strategy with stepwise changing feed rate.

Mixed feeding strategies in Mut^+ *pichia* fermentations (Hellwig et al., 2001; Katakura et al., 1998; McGrew et al., 1997), similar to those for Mut^s strains were reported. Katakura et al. determined that product formation was dramatically improved by simultaneously feeding glycerol at a feed rate of 5 mL/L/h while maintaining 0.55% (v/v) of residual methanol in the induction phase. McGrew et al. employed mixed feeds at a glycerol:methanol ratio of 1:1 and approximately doubled growth and CD40 ligand expression levels, compared to feeding methanol alone. In opposition, Hellwig et al. found that, while maintaining 0.5% of methanol concentration in the induction phase, supplementary feeding of

glycerol strongly inhibited the production of scFv antibody fragment, and expression was almost completely inhibited when the specific glycerol feed rate was higher than 6 mg/mg-WCW/h. Even at glycerol feed rates below 6 mg/mg-WCW/h, the expression level only showed half of that found in the fermentation with methanol feed alone. These attempts at running mixed feeding strategy in Mut^+ *Pichia* fermentations did not further investigate the interaction of cells growth on the two substrates. The objective of this paper is to study mixed feeding strategies based on cell growth kinetics to determine the potential of improving product formation. We will use a GS115 Mut^+ *Pichia* strain as a model system that intracellularly expresses the heavy-chain fragment C of botulinum neurotoxin serotype C [BoNT/C(Hc)]. When growing on methanol, the BoNT/C(Hc) strain showed a maximum specific growth rate (μ_m) of around 0.02 h^{-1} (as shown later), distinguishing it from a value 0.07 h^{-1} obtained for another Mut^+ *Pichia* strain expressing a similar protein, BoNT/A(Hc) (Zhang et al., 2000) and a Mut^s strain we measured to be 0.008 h^{-1}, data not shown. Therefore, choosing BoNT/C(Hc) strain for this study could be representative for both Mut^s and Mut^+ *Pichia* expression systems. The strain was constructed by United States Army Medical Research Institute of Infectious Diseases (USAMRIID). BoNT/C(Hc) is one of the seven serotypes designated types A through G of BoNT(Hc) that corresponds to seven different strains of *Clostridium botulinum*. The recombinant BoNT(Hc) are nontoxic 50 kDa fragments and have been shown to elicit significant protective immunity in mice and are vaccine candidates against the botulinum neurotoxin (Byrne et al., 1998; Clayton et al., 1995).

MATERIALS AND METHODS

Fermentation

A 1-L shake flask with 300 mL BMGY medium (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 4×10^{-5}% biotin, 1% glycerol, 100 mM potassium phosphate, pH 6.0) was inoculated with 1 mL of the stock seed. The cultivation lasted for 20 – 24 hours at 30°C and 300 rpm (shake rate) to reach an optical density (at 600 nm) of 10 – 20. The entire 300 mL propagated culture was used for inoculating a 5-L fermentor (New Brunswick Scientific Co., Edison, NJ) containing 2 L basal salts medium (BSM) and 8.7 mL PTM_1 trace salts. 1 L BSM consists of 26.7 mL 85% H_3PO_4, 0.93 g $CaSO_4$, 18.2 g K_2SO_4, 14.9 g $MgSO_4\cdot7H_2O$, 4.13 g KOH, and 40.0 g glycerol. 1 L PTM_1 (by filter sterilization) consists of 6.0 g $CuSO_4\cdot5H_2O$, 0.08 g NaI, 3.0 g $MnSO_4\cdot H_2O$, 0.2 g $Na_2MoO_4\cdot2H_2O$, 0.02 g H_3BO_3, 0.5 g $CoCl_2$, 20.0 g $ZnCl_2$, 65.0 g $FeSO_4\cdot7H_2O$, 0.2 g biotin and 5.0 mL H_2SO_4. The fermentation was run in fed-batch mode at 30°C, and pH was maintained at 5.0 using undiluted (28%) ammonium hydroxide. The dissolve oxygen (DO) was maintained above 20% of saturation by adjusting agitation rate and pure oxygen supply.

When the initial glycerol (40 g/L) in batch phase was depleted, which is indicated by an abrupt increase of DO reading, a 63% (w/v) glycerol solution containing 1.2% (v/v) PTM_1 was fed at a feed rate of 12 mL/h/L-broth for 1 hour. Then 4 mL methanol was injected into the fermentor, and simultaneously, the glycerol feed rate was programmed to decrease linearly from 12 mL/h/L to 0 over a period of 3 hours. This 3-hour period was considered as a transition phase that is important for cells to adapt the methanol

efficiently and completely (Zhang et al., 2000). Following the transition phase, the production phase started in which 100% methanol containing 1.2% (v/v) PTM_1 and 0.05% (w/v) antifoam (KFO 673, KABO Chemicals Inc., WY) was fed under control of a methanol sensor, or at a programmed feed rate based on a desired growth rate (feeding strategy as shown later). Five or six time-course samples, taken every 4 – 6 hours, were removed during the production phase.

To determine the growth model on glycerol phase, a 63% (w/v) glycerol solution containing 1.2% (v/v) PTM_1 was fed at a programmed feed rate based on a desired growth rate (glycerol feeding strategy as shown later), after the depletion of initial glycerol batch phase. Four to six time-course samples, taken every 3 – 5 hours, were removed during the glycerol fed-batch phase.

Analytical methods

Cell density was expressed as grams of wet cell weight (WCW) per liter broth, which was obtained by centrifuging the samples at 2,000*g* for 10 minutes. All kinetic calculations were based on WCW. Methanol concentration in samples was measured by gas chromatography (GC-17A, Shimadzu Co., Columbia, MD) with isopropyl alcohol as an internal standard.

BoNT/C(Hc) protein was released from cells through bead breaking lysis, and quantified by Western Blot analysis. Cell paste samples of 0.2 – 0.5 g were taken and suspended in 10 mL washing buffer (145 mM NaCl, 31.5 mM sodium acetate, 18.5 mM acetic acid, pH 5.0), then spun at 2,000*g* for 10 minutes to obtain washed cell pellets. The cell pellets were resuspended in lysis buffer (2.5 g/L CHAPS, 5 mM EDTA, 500 mM NaCl, 50 mM NaH_2PO_4, 1 mM PMSP, pH 7.5) to a density of 50 g-WCW/L. 1 mL of the cell suspension was mixed with approximately 2.2 g zirconia/silica beads (Biospec Products, Inc., Bartlesville, OK) in 2.0 mL screw cap tubes followed by disruption at 4°C with a vibrating disrupter (Mini-BeadBeater-8, Biospec Products, Inc., USA) for 8 cycles (1 minute vibrating and 4 minutes resting in each cycle). The lysate/bead mixture was centrifuged until the supernatant was clear.

The acquired supernatant was diluted 3-fold with lysis buffer, and 90 µL of the diluted supernatant was mixed with 30 µL 4X concentrated tris-glycerine SDS sample buffer (252 mM Tris HCl, 40% glycerol, 8% SDS, 0.01% bromophenol blue, pH 6.8). The mixture was heated at 90°C for 5 – 10 minutes, then 35 µL aliquots were loaded onto a 10-well 4-20% Tris-glycine (Novex Pre-Cast gel, Invitrogen Co., San Diego, CA) SDS-PAGE gel. Each sample was loaded in duplicate. A standard sample containing 703.13 ng BoNT/C(Hc) was loaded on each gel. After running the SDS-PAGE (150 volts, 1.8 h, running buffer: SepraBuff™ TRIS GLY SDS Running Buffer, OWL Seperation Systems, Woburn, MA), the gel was soaked in a transfer buffer (SepraBuff™ TRIS GLY Running and Blotting Buffer) for 5 – 10 minutes, then transfer of proteins from the gel to a PVDF membrane (soaked in MeOH before use) was done using a Semi Dry Transfer Cell (Trans-Blot SD, BIO-RAD Laboratories. 12 volts, 1 h). Following transfer, the PVDF membrane was soaked in blocker solution (5% blotting grade dry milk in 100 mL TBS buffer: 25 mM Tris Base, 140mM NaCl 2.5 mM KCl) for 1 h. The membrane was incubated with the primary antibody solution for 16 h (Botulism Antitoxin Standard, Equine Derived Antitoxin to Type C,

USAMRIID, Fort Detrick, MD. 0.5 mg dissolved in 1 mL 50% glycerol, then diluted 1000-fold with the blocker solution), then rinsed in TBS buffer 3 times, each 10 minutes, and soaked in secondary antibody solution for 2 – 4 h (Peroxidase-labled affinity purified antibody, goat anti-horse IgG(H+L), Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD. 0.5 mg dissolved in 1 mL 50% glycerol, then diluted 500 to 1000-fold with the blocker solution). The final rinse was in TBS buffer for 3 times, each 10 minutes to complete the process.

The membrane was visualized by chemiluminescence using ECL+Plus (Lumigen Inc., Southfield, MI) with a 5 minute incubation (in dark room), then exposed onto two films (Hyperfilm™ECL™, Amersham Pharmacia Biotech, Piscataway, NJ) each with different exposure time (2 – 5 minutes). The films were developed (Kodak GBX fixer and developer) to show the bands of BoNT/C(Hc) protein. Band intensities were obtained through scanning the films (HP ScanJet 6300C scanner) and digitizing the bands (UN-SCAN-IT, Automated Digitizing System, Version 5.1, Silk Scientific Corporation, Orem, Utah). BoNT/C(Hc) in each band was quantified from a standard curve that showed the relationship between band intensity and protein amount. Fig. 1 shows the bands of standard samples (Set 1 and Set 2) and the standard curves obtained. Set 3 was from a gel loaded with fermentation samples (lane 3 to 10) and one BoNT/C(Hc) standard (lane 1, 703.13 ng). Set 1 and Set 2 were developed from the same blotted membrane but had different exposure times. It was found that a linear standard curve was obtained and the exposure time did not affect the linearity, which is described as:

$$L_{Set1} = 0.00236 I_{Set1} + 86.3 \qquad (1)$$

$$L_{Set2} = 0.00184 I_{Set2} + 68.5 \qquad (2)$$

where L is the loaded amount of BoNT/C(Hc), ng, and I band intensity, pixel. The effect of exposure time on intensity can be calibrated with a coefficient k, that is, both Eq. (1) and (2) can be expressed as:

$$L = k(aI + b) \qquad (3)$$

Eq. (1) or (2) can be used to determine the values of a and b, e.g., if we employ Eq. (2), a = 0.00184, b = 68.5. In this case, Set 1 has k = 1.27, Set 2 k = 1. For each film with different exposure time, there is a corresponding value of k to calculate the loaded BoNT/C(Hc) amount with Eq. (3). Since there was a standard BoNT/C(Hc) of 703.13 ng loaded on each gel, the k can be calculated as:

$$k = L_{std} / (aI_{std} + b) \qquad (4)$$

where L_{std} is the loaded amount of standard BoNT/C(Hc); I_{std} is the intensity of the band corresponding to the L_{std} = 703.13 ng. With k and I known, the BoNT/C(Hc) amount in loaded samples can be calculated by Eq. (3), and the BoNT/C(Hc) content in cells, α, is obtained as:

$$\alpha = cL/(de) \qquad (5)$$

where c is dilution time of loaded lysate; d cell suspension density for lysis; and e loaded volume of diluted lysate per lane. In this research c = 3, d = 50 g-WCW/L, and e = 26.25 μL. The error of α determined with this quantitation method was within 20% (data not shown).

RESULTS AND DISCUSSION

Growth Kinetics on Glycerol

In the batch phase, with an initial glycerol of 40 g/L, cells were grown under unlimited conditions. The maximum growth rate on glycerol, $\mu_{gly,m}$, and maximum glycerol specific consumption rate, $\nu_{gly,m}$, were determined, i.e., $\mu_{gly,m} = 0.177\ h^{-1}$ and $\nu_{gly,m} = 0.0688$ g/h/g-WCW. Knowing $\mu_{gly,m}$ and $\nu_{gly,m}$, the glycerol feed rate, F_{gly}, can be estimated to run a fed-batch process with the desired $\mu_{gly,d}$ ($\leq \mu_{gly,m}$) by:

$$F_{gly} = \frac{\mu_{gly,d}\upsilon_{gly,m}X_0V_0e^{\mu_{gly,d}t}}{\mu_{gly,m}} \qquad (6)$$

where X_0 and V_0 are the cell density and broth volume at the beginning of the glycerol fed-batch phase. Fed-batch runs with different $\mu_{gly,d}$ from 0 to $\mu_{gly,m}$ were conducted using the feeding strategy, Eq. (6). The actual μ_{gly}, which could be different from $\mu_{gly,d}$, ν_{gly} and $\nu_{NH3(gly)}$ (ammonium specific consumption rate) were determined from each run. The method to calculate the specific rates (as well as all other kinetics) was referred to in a previous publication (Zhang et al., 2000). Fig. 2 shows the linear dependence of ν_{gly} and $\nu_{NH3(gly)}$ on μ_{gly}, and the resulting equations:

$$\upsilon_{gly} = 0.503\mu_{gly} + 0.0065 \qquad (7)$$

$$\upsilon_{NH3(gly)} = 0.0852\mu_{gly} + 0.0007 \qquad (8)$$

With the fed-batch growth model Eq. (7), estimation of F_{gly} by Eq. (6) was corrected to realize a growth rate more approaching $\mu_{gly,d}$, namely:

$$F_{gly} = (0.503\mu_{gly,d} + 0.0065)X_0V_0e^{\mu_{gly,d}t} \qquad (9)$$

As shown later, the feeding strategy of Eq. (9) will be applied to the design of fed-batch fermentation with mixed feeds of glycerol and methanol.

The catabolic pathway of glycerol in methylotrophic yeast, such as *Pichia*, involves passive diffusion across the membrane, phosphorylation by a glycerol kinase, and oxidation by a mitochondrial glycerol phosohate ubiquinone oxireductase (Gancedo et al., 1968). Glycerol enters glycolysis after its conversion to glyceraldehyde 3-phosphate, and requires respiration to dispose of NADH in order to serve as an energy source. The whole glycerol catabolic process is independent of the methanol metabolism pathways described elsewhere (Sibirny et al., 1988). Therefore, although the growth model on glycerol, Eq. (7) and (8), was obtained with the BoNT/C(Hc) strain employed in this research, the model can be applied to the design of fed-batch growth on glycerol for all other GS115 derived *Pichia* clones, regardless if they are Mut^+ or Mut^s.

Growth Kinetics on Methanol

It is known that *Pichia pastoris* can not tolerate high methanol concentration in the fermentation due to the accumulation of formaldehyde and hydrogen peroxide inside the cells, both of which are the oxidized products of methanol by the alcohol oxidase and are toxic to the cells (Couderc and Baratti, 1980; Cregg and Madden, 1988; Van der Klei et al., 1990). As discovered in our previous work (Zhang et al.,

2000), methanol levels above 3.65 g/L (a value calculated from the growth model) start to show growth inhibition. Therefore, unlike the growth on glycerol, it is impossible to measure the maximum growth rate ($\mu_{MeOH,m}$) by growing cells in batch mode with a high methanol level. To reveal the $\mu_{MeOH,m}$ as well as the corresponding maximum methanol specific consumption rate ($\nu_{MeOH,m}$), we ran the fed-batch fermentation employing a methanol sensor (MC-168 Methanol Monitor and Controller, PTI Instruments Inc, Kathleen, GA. The sensing material in the MC-168 is TGS822 alcohol sensor from Figaro USA Inc, Glenview, IL) to control the methanol feeding to maintain the methanol level at 2 – 4 g/L.). A proportional, integral and derivative (PID) control mode was applied for the methanol control system (Zhang et al., 2001). Fig. 3 shows the time course of total cell growth (XV) and the methanol level (S) controlled by the sensor. Exponential growth was observed within 50 h of methanol feeding time. The $\mu_{MeOH,m}$ was 0.02 h^{-1}, and $\nu_{MeOH,m}$ 0.028 g/h/g WCW. The $\mu_{MeOH,m}$ is much lower than that of a typical Mut^+ *Pichia* strain, i.e., 0.07 h^{-1} (Zhang et al., 2000), but higher than a Mut^s strain, 0.008 h^{-1}, data not shown. This suggests that methanol metabolism of the BoNT/C(Hc) *Pichia* strain was affected either by expression of the heterologous protein or the gene insertion event. The similar phenomenon was also observed in a *E. coli* expression system in which a recombinant protein changed host strain bioactivities (Wong et al., 1998).

Based on the obtained $\mu_{MeOH,m}$ and $\nu_{MeOH,m}$, we conducted fed-batch fermentations with different desired growth rates, $\mu_{MeOH,d}$ ($\leq \mu_{MeOH,m}$), by feeding methanol at a feed rate, F_{MeOH}, estimated as:

$$F_{MeOH} = \frac{\mu_{MeOH,d}\upsilon_{MeOH,m}X_0V_0e^{\mu_{MeOH,d}t}}{\mu_{MeOH,m}} \qquad (10)$$

where X_0 and V_0 are the cell density and broth volume at the beginning of the methanol fed-batch phase. Similar to the limited fed-batch growth on glycerol, an actual μ_{MeOH}, ν_{MeOH} and $\nu_{NH3(MeOH)}$ were obtained from each of the runs, and the linear dependence of ν_{MeOH} and $\nu_{NH3(MeOH)}$ on μ_{MeOH} are shown in Fig. 4. The linear relationships are expressed as:

$$\upsilon_{MeOH} = 0.766\mu_{MeOH} + 0.0128 \qquad (11)$$

$$\upsilon_{NH3(MeOH)} = 0.153\mu_{MeOH} \qquad (12)$$

Eq. (11) includes the maintenance coefficient, 0.0128 g-MeOH/h/g-WCW, and was substituted into Eq. (10), to give Eq. (13):

$$F_{MeOH} = (0.766\mu_{MeOH,d} + 0.0128)X_0V_0e^{\mu_{MeOH,d}t} \qquad (13)$$

Mixed Feed and Production Improvement

The BoNT/C(Hc) content in the cells (α) reached a maximum after 10 h of methanol feeding, and remained constant during the remainder of exponential growth phase, similar to the intracellular production of BoNT/A(Hc) (Zhang et al., 2000). The average of α during the stable period (defined as quasi-steady state) represented the production level at a corresponding μ_{MeOH}. The effect of μ_{MeOH} on α using only MeOH during fed-batch is presented in Fig. 5. The optimum μ_{MeOH} was 0.015 h^{-1} and achieved an α of 2

mg/g. This is similar to that observed for BoNT/A(Hc) production (Zhang et al., 2000) in which the optimal μ_{MeOH} was 0.0267 h^{-1} rather than the $\mu_{MeOH,m}$ (0.0709 h^{-1}).

The detailed mechanism of why μ affects α remains unknown, but the imbalance of energy supply for growth and production is believed to be one of the reasons (Chim-Anage et al., 1991; Shioya, 1992). Based on this supposition and considering the slow methanol assimilation in this strain, we proposed a mixed feeding strategy, namely, feeding glycerol simultaneously in methanol fed-batch phase, to explore the potentiality of improving the production. As we discussed, the mixed feeding strategy is generally employed for Mut^s *Pichia* fermentations in view of its slow utilization of methanol, and was also applied in Mut^+ strains by several researchers. However, all these studies relied on arbitrary combinations of the two substrates (glycerol and methanol) for optimizing the mixed feed design. Here we investigated the strategy based on the growth kinetics, explicitly, we optimized the growth rate ratio of $\mu_{gly(mix)}$:$\mu_{MeOH(mix)}$ rather than the quantity ratio of glycerol:methanol in the mixed feed. $\mu_{gly(mix)}$ and $\mu_{MeOH(mix)}$ are the growth rate contributed by glycerol and methanol, respectively, and the total growth rate on a mixed feed, μ_{mix}, expressed as:

$$\mu_{mix} = \mu_{gly(mix)} + \mu_{MeOH(mix)} \qquad (14)$$

We ran a mixed feeding strategy with desired μ_{gly}:μ_{MeOH} = 1, 2, 3, 4 to study the growth interaction of the two substrates and its effects on the production. The growth on methanol feed alone, μ_{MeOH} = 0.015 h^{-1} was the optimal growth rate for maximum α. Accordingly, we fixed the desired growth rate on methanol ($\mu_{MeOH(mix),d}$) at 0.015 h^{-1} while varying the desired growth rate on glycerol ($\mu_{gly(mix),d}$) for the mixed feed design. Based on Eq. (9) and (13), the glycerol and methanol feed rate were given as:

$$F_{gly(mix)} = (0.503\mu_{gly(mix),d} + 0.0065)X_0V_0e^{\mu_{mix,d}t} \qquad (15)$$

$$F_{MeOH(mix)} = (0.766\mu_{MeOH(mix),d} + 0.0128)X_0V_0e^{\mu_{mix,d}t} \qquad (16)$$

where $\mu_{mix,d}$ was calculated as $\mu_{MeOH(mix),d}$ + $\mu_{gly(mix),d}$ according to Eq. (14). Fig. 6 shows the actual μ_{mix} achieved from the runs performing the feeding strategy of Eq. (15) and (16) with $\mu_{MeOH(mix),d}$ = 0.015 h^{-1} and various $\mu_{gly(mix),d}$. It was found that μ_{mix} was slightly higher than $\mu_{mix,d}$ and had a relationship with $\mu_{gly(mix),d}$ as:

$$\mu_{mix} = 1.18\mu_{gly(mix),d} + 0.015 \qquad (17)$$

Compared with Eq. (14) and noting $\mu_{MeOH(mix),d}$ = 0.015 h^{-1}, Eq. 17 shows that the total growth was promoted in the presence of glycerol feeding. It is known that excess glycerol inhibits the *AOX* promoter (Tschopp et al., 1987). Here we obtained an opposite result due to the fact that the glycerol feed rate we ran only supported a growth below 0.06 h^{-1}, which was far from the maximum growth rate on glycerol (0.177 h^{-1}). Thus, we concluded that in the growth on a mixed feed with $\mu_{gly(mix),d} \leq 0.06$ h, i.e. $\mu_{gly(mix),d}$:$\mu_{meOH(mix),d}$ ≤ 4 for this strain, the supplementary feeding of glycerol enhanced the overall growth rather than functioned as a repressor. This observation indicates that running a mixed feed in Mut^+ *Pichia* fermentations is feasible without causing growth inhibition by glycerol when a feeding strategy is properly

designed. The production level under the mixed feed design can be looked at to discover the optimal feeding strategy.

Fig. 5 shows the production levels obtained under the mixed feed strategy with various $\mu_{gly(mix),d}/\mu_{meOH(mix),d}$ while $\mu_{MeOH,d}$ was set to 0.015 h^{-1}, as well as a comparison with methanol feed alone. It was found that $\mu_{gly(mix),d}/\mu_{meOH(mix),d} = 2$, which corresponded to an obtained μ_{mix} of 0.05 h^{-1}, appeared to deliver the highest α, and the production was not inhibited by the supplementary glycerol feeding until a feeding strategy with $\mu_{gly(mix),d}/\mu_{meOH(mix),d}$ higher than 3 was run. Substituting the optimal $\mu_{gly(mix),d} = 0.03$ h^{-1} and $\mu_{meOH(mix),d} = 0.015$ h^{-1} into Eq. (15) and (16), the optimal feed rate ratio of $F_{gly(mix)}$:$F_{MeOH(mix)}$ was simply derived to be 0.889. This discovery demonstrated that the mixed feeding strategy based on the growth kinetics as Eq. (7) and (11) can be optimized to maximize production. The optimal strategy can be applied to any cell densities and easily scaled-up to any size of fermentation as a result of the direct association with growth rate instead of the arbitrary combinations of the two substrates that was used in previous research. Since the growth models on both glycerol [Eq. (7) and (8)] and methanol [Eq. (11) and (12)] were developed, a thorough simulation for the optimal process can be made in the same way as what we did in the BoNT/A(Hc) production optimization (Zhang et al., 2000). It is predicted that the methods developed in this paper can be applied to mixed feed design for both Mut^+ and Mut^s *Pichia* fermentations producing a variety of recombinant proteins.

ACKNOWLEDGEMENTS

We thank UNL undergraduate students Kian Ann Teh and Kok Wah Lim for operating the fermentations, and Michael Dux and Angeline Yong for performing the Western Blot analysis. We thank Jacqueline D. Andersen and all other members of BPDF-UNL fermentation group for their assistance in running the fermentations. This research was financially supported by the United States Army Medical Research and Materiel Command (contract number: DAMD-17-98-C-8034).

NOMENCLATURE

AOX	alcohol oxidase
BoNT/C(Hc)	heavy-chain fragment C of botulinum neurotoxin serotype C
F	substrate feed rate, g/L
I	intensity of band from Western Blot, pixel
L	loaded amount of BoNT/C(Hc) in SDS-PAGE, ng
Mut^+, Mut^s	methanol utilization plus and slow phenotypes
V	fermentation broth volume, L
WCW	wet cell weight, g
X	cell density, g WCW/L
α	BoNT/C(Hc) content in cells, mg/g WCW
μ	specific growth rate, h^{-1}
ν	substrate specific consumption rate, g/h/g WCW

Subscripts

d	desired value
gly	kinetic parameter related to the growth on glycerol
m	maximum value
MeOH	kinetic parameter related to the growth on methanol
mix	kinetic parameter related to the growth on mixed feeds of glycerol and methanol
NH3	kinetic parameter related to the consumption of ammonium (28% NH_3)
0	initial time point of glycerol or methanol fed-batch phase

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LIST OF FIGURE

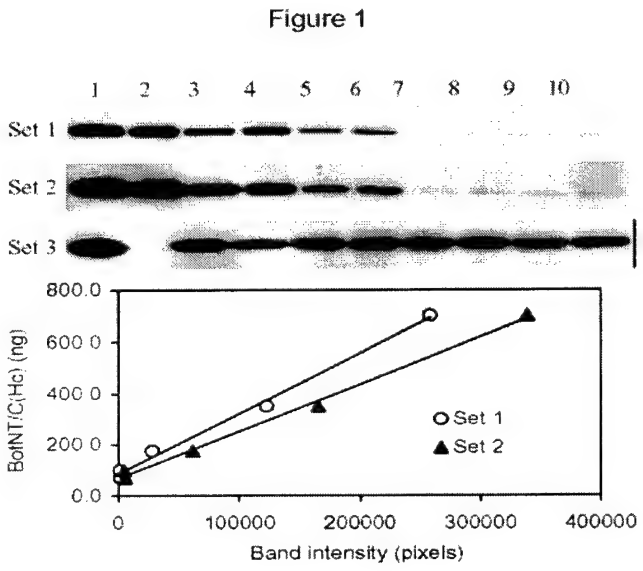


Figure 1. Western Blot bands of BoNT/C(Hc) standard samples and the standard curve for relationship between protein amount and band intensity. Loaded amount (ng) for Set 1 and 2: Lane 1 and 2, 703.13; 3 and 4, 351.56; 5 and 6, 175.78; 7 and 8, 100.45; 9 and 10: 70.31. Set 1 and Set 2 was developed from the same blotted membrane but different exposure time. Set 3 shows bands from a gel loaded with fermentation samples (lane 3 to 10), one BoNT/C(Hc) standard (lane 1, 703.13 ng), and molecule markers (lane 2).

Figure 2

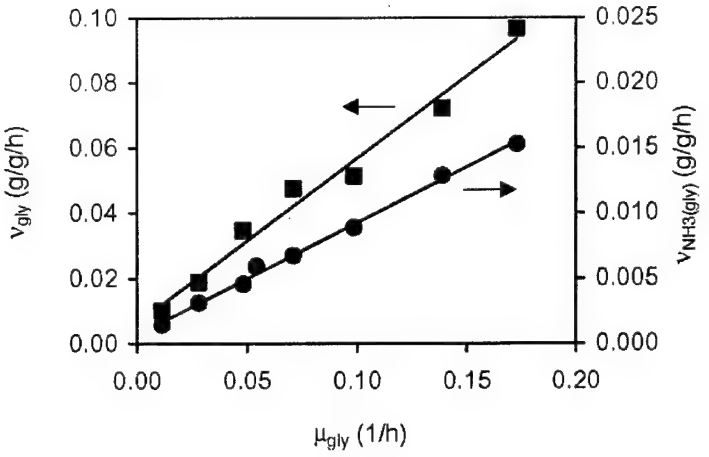


Figure 2. Dependences of glycerol and ammonium specific consumption rates on specific growth rate in glycerol fed-batch growth.

Figure 3

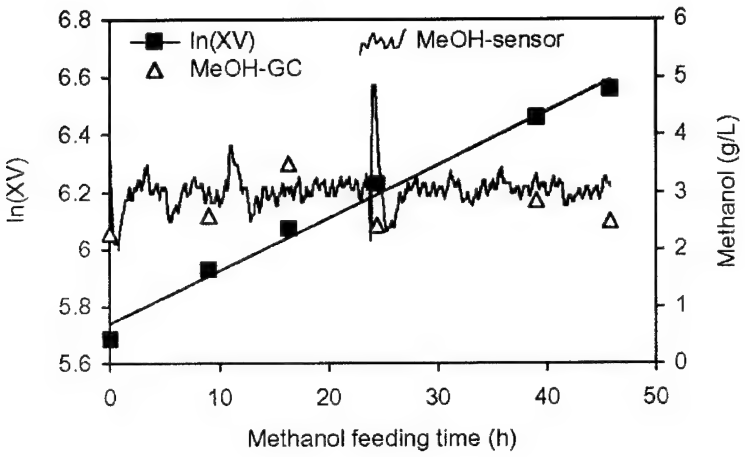


Figure 3. Time course of total grown cells and controlled methanol level in methanol fed-batch phase. MeOH-sensor: methanol on-line read value from the methanol sensor, MeOH-GC: methanol concentration in the samples analyzed with gas chromatography.

Figure 4

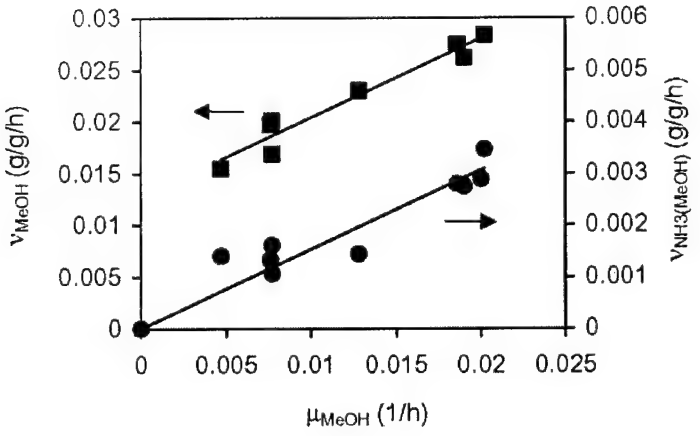


Figure 4. Dependences of methanol and ammonium specific consumption rates on specific growth rate in methanol fed-batch growth.

Figure 5

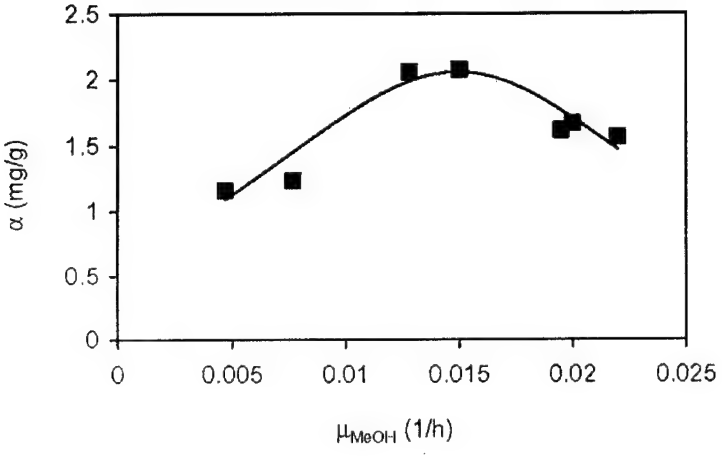


Figure 5. BoNT/C(Hc) content in cells (α) under methanol feed alone and mixed feed.

Figure 6

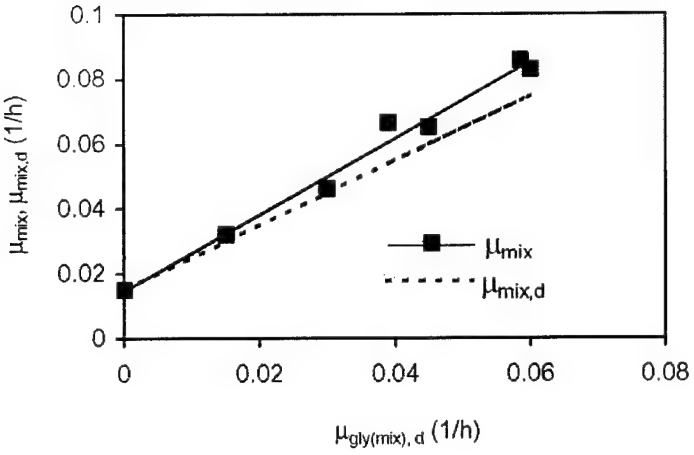


Figure 6. Actual μ_{mix} achieved from the runs with $\mu_{MeOH(mix),d} = 0.015\ h^{-1}$ and various $\mu_{gly(mix),d}$. $\mu_{mix,d} = \mu_{MeOH(mix),d} + \mu_{gly(mix),d}$ was also plotted as dotted line.

BoNTC Hc Purification process

The following purification process produced, on average, 120mg BoNT-C/kg cell paste with 95% purity based on SDS-PAGE. The final Poros PI 50 column results in two BoNT-C peaks: peak 1 consisting of BoNTC Hc missing the first eight amino acids of N-terminus and peak 2 consisting of a shorter form, although it has the same N-terminus. Over time it has been seen that peak 2 degrades into a 30kDa fragment that lights up by Western analysis.

Cell washing

Sixty g cell paste (frozen –20C) from BoNTC Hc fermentation WZ 143 (10h induction) was dissolved to 20% (w/v) solids in cold breaking buffer (50mM sodium phosphate, pH 7.5 + 0.25%CHAPS + 5mM EDTA + 0.5M sodium chloride + 1mM PMSF). This mixture was separated by centrifugation at 12,400 x g for 10 min @ 4C. The resulting pellet (~ 44g) was saved as "cell pellet" for further processing.

Cell disruption

Forty g cell pellet was mixed to 5% (w/v) in cold breaking buffer (50mM Sodium Phosphate, pH 7.5 + 0.25%CHAPS + 5mM EDTA + 0.5M Sodium Chloride + 1mM PMSF). This material was passed four to five times through a Microfluidics M-110EH microfluidizer at 23,500 psi, resulting in a typical breakage of 85% based on Hemocytometer results. During processing the temperature ranged from 3 to 12°C. The homogenate was centrifuged at 12,400 x g for 30 min @ 4°C (JA-10, 8,500rpm). The supernatant was adjusted to 0.25% polyethylinimine (PEI) using a 10% PEI stock in 50mM Sodium Phosphate, pH 7.5 at which point a ppt. forms. The mixture was immediately adjusted to pH 6.0 using 5N HCl and mixed at room temperature for 45 minutes. The solution was clarified by centrifugation at 12,400 x g for 30 min @ 4C. Pellets from both the pre- and post-PEI treatment show BoNT-C present by Western analysis (see below).

Diafiltration

PEI-treated supernatant was diafiltered using a 2 sq. ft. 30K MWCO PES Triport cassette (NCSRT) at 0.75-1.0m/s, 15-20psi, 21 +/- 2°C with Q Sepharose equilibration buffer (50mM sodium phosphate, pH 7.5 + 0.2% CHAPS, 5mM EDTA + 1mM PMSF). The diafiltration was continued until three criteria were met by the retentate; 1) UV 260/280 <1.0, 2) pH =7.5 +/-0.1, and 3) Conductivity </= 8.3 mS @21 +/-2°C, which requires at least four diafiltration volumes. During the diafiltration, a significant ppt forms, which does not contain BoNTC Hc based on Western analysis of the pellet. At this point in the process, the retentate was allowed to sit overnight packed on ice in a 4°C cold room. The retentate was clarified by centrifugation at 14,000 x g for 25 min @ 4°C (JA-10, 9,000rpm). The supernatant was passed through a 0.2um PES filter prior to chromatographic processing.

Q Sepharose FF Chromatography

The clarified product (chilled on ice) was loaded onto a 100mL (2.6 x 20cm) Q Sepharose FF column (14 mg total protein/mg resin) at 100 cm/h. The column was equilibrated with 10 column volumes (CV) of Q equilibration buffer (50mM sodium phosphate, pH 7.5 + 0.2% CHAPS, 5mM EDTA + 1mM PMSF) at 300 cm/h. After loading, the column was washed with 12 CV Q Equilibration buffer at 300cm/h.

To elute BoNTC Hc, a 10CV linear gradient was performed from 0-0.5M Sodium Chloride. BoNTC Hc elutes as the second of three broad peaks at ~125mM sodium chloride.

ToyoPearl Phenyl 650M Chromatography

Q Sepharose FF product pool (~200mL) was adjusted to 1M ammonium sulfate using the granular form. The solution was mixed at room temperature for 30 minutes. There was no significant ppt formed, but the material was passed through a 0.2um PES filter to remove dust/debris particles. This material was loaded onto a 26mL (1.6 x 13 cm) ToyoPearl Phenyl 650M column (2mg total protein/mL resin) at 300cm/h. Prior to loading, the column was equilibrated with 10 CV HIC equilibration buffer (1M ammonium sulfate + 50mM sodium phosphate + 2mM EDTA, pH 7.5). The column was washed with 7 CV HIC equilibration buffer, and the product was eluted using a 10 CV linear gradient to 50mM sodium phosphate + 2mM EDTA, pH 7.5. BoNTC Hc elutes as the third peak at the end of the gradient.

Poros PI 50 Chromatography

HIC product pool (~90mL) was dialyzed using 12-14K MWCO SpectraPor dialysis tubing at 4°C versus 15 mM Tris + 2mM EDTA, pH 8.0 overnight. This material was then polished using an 8mL (1 x 10cm) Poros PI 50 column at 750cm/h. The column was pre-equilibrated with PI equilibration buffer (15mM Tris + 2mM EDTA, pH 8.0) at 750cm/h. The dialyzed HIC product was loaded at 1.4mg total protein/mL resin and was ~75% pure prior to the PI step. After loading, the column was washed with 10 CV PI equilibration buffer and stepped to 30mM Histidine + 2mM EDTA, pH 6.5 for 5 CV. Product was then eluted by a 20 CV linear pH gradient to 15mM sodium acetate + 2mM EDTA, pH 4.0. BoNTC Hc elutes right as the pH reaches 5.0. Two peaks elute during this step, both containing BoNTC Hc.

Final buffer exchange

Both peaks 1 and 2 from the PI column were dialyzed into 50mM Sodium Phosphate + 50mM Sodium Chloride, pH 7.5 using SpectraPor 12-14 MWCO tubing or Slide-A-Lyzer 10K MWCO cassettes overnight at 4°C. The resulting material was then stored at 4C for about two weeks and then snap frozen and stored at –20°C.

Results

On average, 120.92mg (143.57 to 104.56) BoNTC Hc was purified per kg cell paste. This value is somewhat low based on past results of other BoNT serotype purifications (150-200mg/kg), and may be due to loss of BoNTC Hc in the harvest and PEI treatment pellets. Without an effective HPLC assay, it is difficult to determine the actual amount of BoNTC Hc lost during these steps. Product generated from the above purification process is at least 95% pure based on SDS-PAGE (Coomassie).

A review of the three runs performed shows an average release of 78 g total protein per kg cells, which is comparable to other BoNT serotype purifications (50-90g/kg). The average % cell breakage (based on Hemocytometer results) was 69. This value is much lower than expected because cells were fresh (harvested four days before being processed) and conflicts with the expected amount of protein released. This may reflect the inaccuracy of the Hemocytometer method.

During initial development of this process, pellet samples of the pre- and post-PEI and post UF were analyzed by Western analysis and showed no BoNTC Hc. Samples were prepared by removing a chunk of pellet and placing it in 2 mL 5M Urea. This method was difficult to reproduce because the amount of pellet was not weighed. Since then, it was decided to weigh 0.05 g of pellet and suspend it in 1 mL 5M Urea. Using this method, BoNTC Hc is present in the pre- and post-PEI pellets. One possibility may be that originally too much protein was being transferred to the membrane, which prevented the primary from binding to BoNTC Hc, thus inhibiting detection. Therefore, we may have been losing BoNT-C in the pellets of these steps all along. The amount lost in these steps is unknown at this time.

It was found over time that BoNTC Hc in both peaks 1 and 2 was degrading to a 30 kDa fragment. This was occurring more so in peak 2. Samples of peak 1 and 2 were both exchanged into pH 4.0 and pH 7.5 and were analyzed by SDS-PAGE to determine if pH had an impact on degradation. Results (not shown) indicated that pH 7.5 showed a greater amount of the 30 kd fragment and that all samples contained intact BoNTC Hc. BoNTC Hc standard (generated from the same *P. pastoris* clone) provided by USAMRIID degrades to a 30 kd fragment if exposed to multiple freeze/thaws.

Samples from run 109-174 final Peak 1 and Peak 2 have identical N-terminal sequences (missing 8 a.a.), suggesting that any difference in sequence may be occurring at the C-terminus.

All buffers up to and through the Q Sepharose FF column contain at least 0.2% CHAPS. Samples have been sent to the analytical department for HPLC analysis to determine how much, if any, CHAPS is still present in the final product. Due to the hydrophobic nature of BoNTC Hc, it is expected that some CHAPS will remain bound to the protein. Results are undetermined at this time.

Future work

To go from 95% to 99% purity, more work is needed on the polishing step. Possible alternatives include 1) including a salt gradient in conjunction with the existing pH gradient on the PI column, 2) heparin-agarose chromatography, or 3) another weak anion exchange column (DEAE, for example). The possible presence of CHAPS with the product will also need to be addressed.

Purification Table

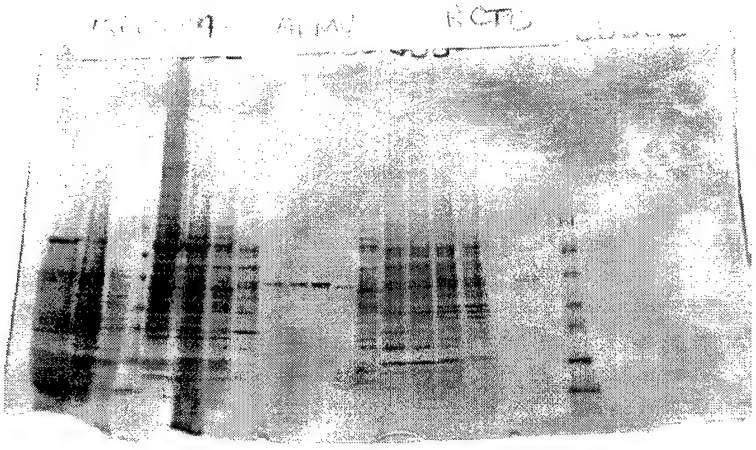
Run #142-4
Broke 44.07g cells

Step	Volume (mL)	[Total Protein] (mg/mL)	Total Protein (mg)	% Step Yield Total Protein	% Total Yield Total Protein
Homogenate	1400	2.73	3822	100	100
PEI treatment	1380	2.23	3077	81	81
UF/DF	1820	1.03	1875	61	49
Q Seph FF	204	0.328	66.91	4	1.75
Phenyl 650M	87	0.171	14.88	22	0.39
Post dialysis	98	0.158	15.48	104	0.41
Poros PI 50 pk1	30	0.222	6.66	43	0.17
Poros PI 50 pk2	26	0.174	4.52	29	0.12

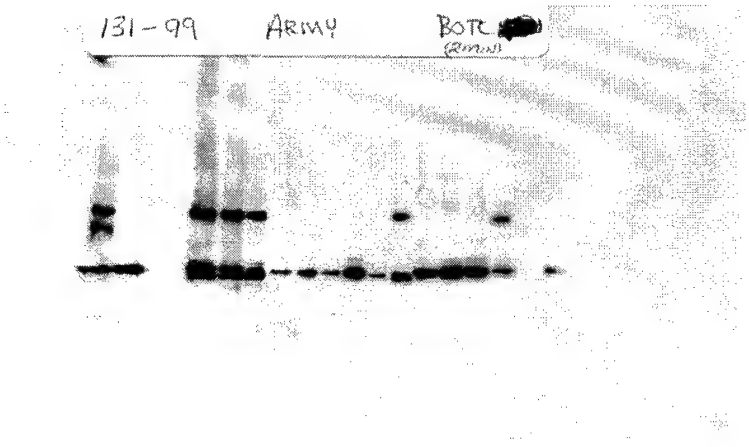
Total Protein released 86.73 g T.P./kg cells
Final BoNT-C yield* 143.57 mg BoNT-C/kg cells

*Assuming 95% purity of peak 1

SDS-PAGE coomassie of in-process samples



Western Blot of in–process samples



Lane	Sample I.D.
1	Pre-PEI pellet
2	Post-PEI pellet
3	Post UF pellet
4	M.W. Standard
5	Pre-PEI
6	Post-PEI
7	Q load
8	Q product
9	HIC product
10	PI load
11	PI peak 1
12	PI peak 2
13	Q end of F.T.
14	Q frac 52
15	Q frac 55
16	Q frac 58
17	Q frac 64
18	PI end of F.T.
19	PI frac 53 (end of peak 2)
20	BoNT-C standard (NOTE: lanes 20 and 21 are flipped in the Western)
21	M.W. standard

4. BoNTE Hc

Fermentation Process Development

The BoNTE Hc fermentation process was not optimized and the only purpose of fermentation was to provide cell mass for protein purification. To meet this requirement the BPDF used the same process as the BoNTA Hc fermentation procedure (Reference). Based on SDS-PAGE and western blot the fermentation process was adequate for purification studies.

Purification Process Development

Cell Disruption

Fresh or frozen cell paste is disrupted by high pressure homogenization at a 20% cell/buffer ratio using cold lysis buffer (50mM sodium phosphate buffer, 1 mM PMSF, pH 7.4). The lysate is centrifuged at 12,800xg for 25 minutes at 4°C to remove cell debris. The supernatant is diluted with lysis buffer to 4 mg/ml total protein concentration, and filtered through a 0.45um filter.

Capture Studies

The greatest challenge with the BoNTE Hc purification development effort was the capture step. Many different resins and conditions were tested to find a condition that would effectively bind BoNTE Hc to the first capture column. Unfortunately, this has been a common theme with all of the BoNT Hc. For example, both BoNTA Hc and BoNTC Hc required CHAPS for the respective molecules to completely bind to the first capture column. In all cases some of the BoNTA or C would bind to the column, but there was always some material that would not bind, typically 40 to 50%. This was viewed as unacceptable for a capture step and, in the case of BoNTE HC, experiments were performed to improve the capture efficiency. What was eventually determined to be the best capture method was batch binding, which is described later in detail in the section under the Final Purification heading. This section, Capture Studies, is dedicated toss all of the different methods that were attempted to increase the binding efficiency of BoNTE Hc to the first column.

Using standard conditions BoNTE Hc did not bind very well under dynamic conditions to different ion exchange resins with 40 to 50% of BoNTE Hc flowing through. Based on past experiences with BoNTA and C Hc it was anticipated that this unusual chromatographic behavior was attributed to the either protein-protein or more likely protein-DNA interaction. CHAPS was used successfully with BoNTA and C Hc, suggesting a DNA-protein interaction. Experiments described below focused on trying to disrupt any type of protein-protein or protein-DNA interaction to facilitate binding to the first capture step. Prior to testing different additives to the cell lysate, a standard cation resin screening was performed.

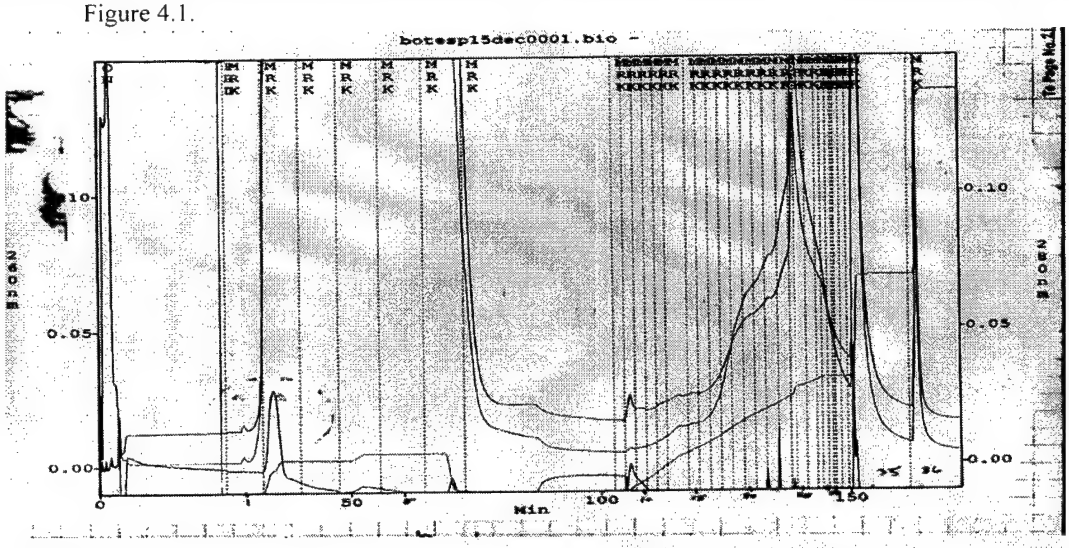
Resin Screening

A study was performed to determine which cation resin was better at binding BoNTE Hc using 50 mM phosphate buffer, pH 7.4. This buffer system was used successfully at USAMRIID to capture BoNTE Hc onto a MonoS analytical resin from Pharmacia Biotech. Four resins were tested, Pharmacia SP-Sepharose FF, BioRad Macro-Prep High S, TosoHaas Toyopearl SP-650S and Applied Biosystems Poros HS 50. All the resins showed a large amount of BoNTE Hc in the flow through, except for Poros HS 50 which had very little in flow through and most in the elution fraction. Though this result was encouraging, there was concern about BoNTE Hc still present in the flow through and using an expensive resin as a capture step. Studies were initiated to determine if the lysate could be altered to better effect binding of BoNTE Hc to the cation exchange column.

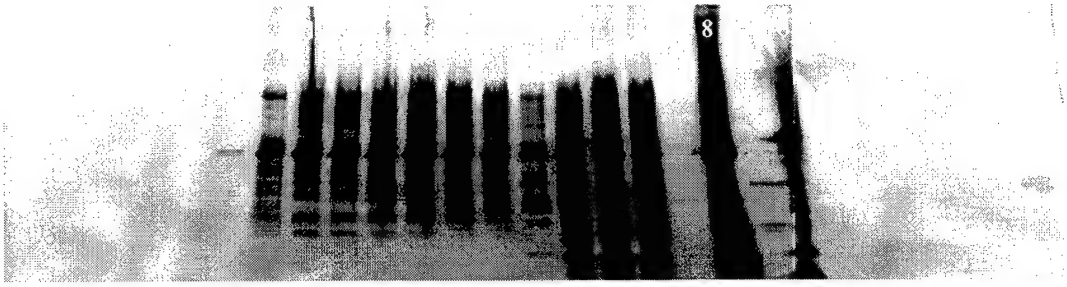
$(NH_4)_2SO_4$ Precipitation

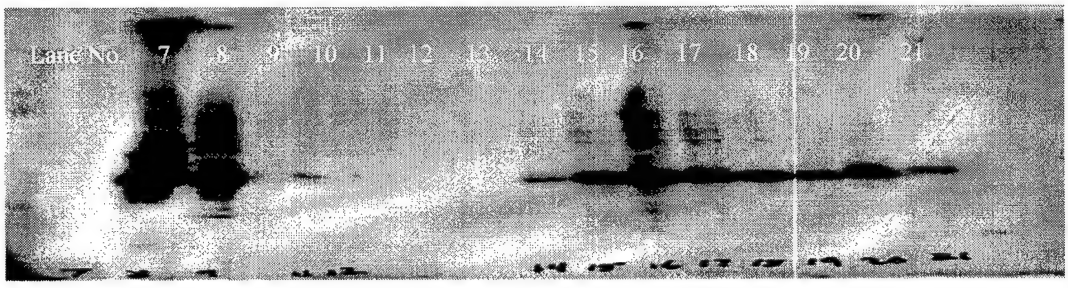
One molar $(NH_4)_2SO_4$ was added to clarified cell lysate in an attempt to remove potentially interfering proteins. The ammonium sulfate supernatant was dialyzed and applied to a SP-Sepharose FF column. There was no BoNTE Hc in the flow through fractions and BoNTE Hc eluted off in the NaCl gradient (Figures 1). Unfortunately, these results could not be repeated, with most of the BoNTE Hc in the column flow through. Also, there was BoNTE in the $(NH_4)_2SO_4$ pellet and in the pellet that formed after

Figure 4.1.



Fraction No. 1 5 10 15 20 25 30 35 36





Lane#6 - SeeBlue MW Standard
Lane#7 - BotE standard
Lane#8 - Load sample
Lane#9 - Fraction #1
Lane#10 - Fraction #3
Lane#11 - Fraction #5
Lane#12 - Fraction#6
Lane#13 - Fraction #18
Lane#14 - Fraction #20
Lane#15 - Fraction #22
Lane#16 - Fraction #24
Lane#17 - Fraction #26
Lane#18 - Fraction #28
Lane#19 - Fraction #30
Lane#20 - Fraction #35
Lane#21 - Fraction #36

dialyzing the ammonium sulfate supernatant into the SP-Sepharose FF loading buffer. Different concentrations of $(NH_4)_2SO_4$ were added to unclarified cell lysate to eliminate a centrifugation step. Again, there was a significant amount of BoNTE Hc in the $(NH_4)_2SO_4$ pellet and the precipitate after dialysis. In an attempt to avoid the precipitation of BoNTE Hc caused by the dialysis step, the supernatant from the $(NH_4)_2SO_4$ precipitation was loaded onto Butyl Sepharose FF column. Unfortunately, BoNTE Hc was present in the flow through.

Due to the presence of BoNTE Hc in the $(NH_4)_2SO_4$ pellet, in the precipitate after dialysis of the $(NH_4)_2SO_4$, and inconsistent binding to the SP-Sepharose FF column it was decided not to pursue this approach any further.

Polyethyleneimine (PEI) Precipitation

PEI was added at 0.25% v/v to clarified (by centrifugation) cell lysate with and without 0.25% CHAPS (v/v). Under both conditions there was BoNTE Hc present in the cell lysis pellet, PEI precipitate pellet and the precipitate after dialysis. It was difficult to determine the amount of BoNTE Hc in each of the pellets, but qualitatively there appeared to be a significant amount in the final supernatants. This experiment was repeated using unclarified supernatant resulting in similar results, suggesting that clarifying the cell lysis was not necessary prior to adding PEI. The supernatants were loaded onto a Poros HS 50 column with most of the BoNTE Hc in the flow through fractions.

Non-Ionic Detergents – IGEPAL CA-630 & Tween 20

IGEPAL CA-630 was added to the lysis buffer at different concentrations (0.01, 0.05, 0.1 & 0.5%). In addition, some samples were treated with either 0.25% PEI or 10% PEG (15-20k). All of the final supernatants, except for the samples treated with 0.05 and 0.1% IGEPAL CA-630 with 0.25% PEI showed very little BoNTE Hc based on western blot (data not shown). The lysis pellets contained BoNTE Hc based on western blot (data not shown). Next, a combination of 0.05% IGEPAL CA-630 and 0.5% Tween 20 with and without 0.3M NaCl in the lysis buffer was evaluated. The clarified supernatants were loaded onto a Poros HS 50 column and a 2-step elution was performed using a 0.5M & 1M NaCl. Most of the BoNTE Hc was in the column flow through.

Final BoNTE Hc Purification Process

Cell Disruption

Fresh or frozen cell paste was disrupted by high pressure homogenization at a 20% cell/buffer ratio using cold lysis buffer (50mM sodium phosphate buffer, 1 mM PMSF, pH 7.4). The lysate was centrifuged at 12,800xg for 25 minutes at 4°C to remove cell debris. The supernatant was diluted with lysis buffer to 4 mg/ml total protein concentration and filtered through a 0.45μm filter.

Purification

All chromatographic purification steps (columns and buffers) were performed at ambient temperature with the column load on ice. Development scale separations were performed using the Applied Biosystems BioCAD chromatography system. Purification was attained via a four-step

chromatographic procedure with the first three steps ion exchange followed by an hydrophobic interaction chromatography polishing step.

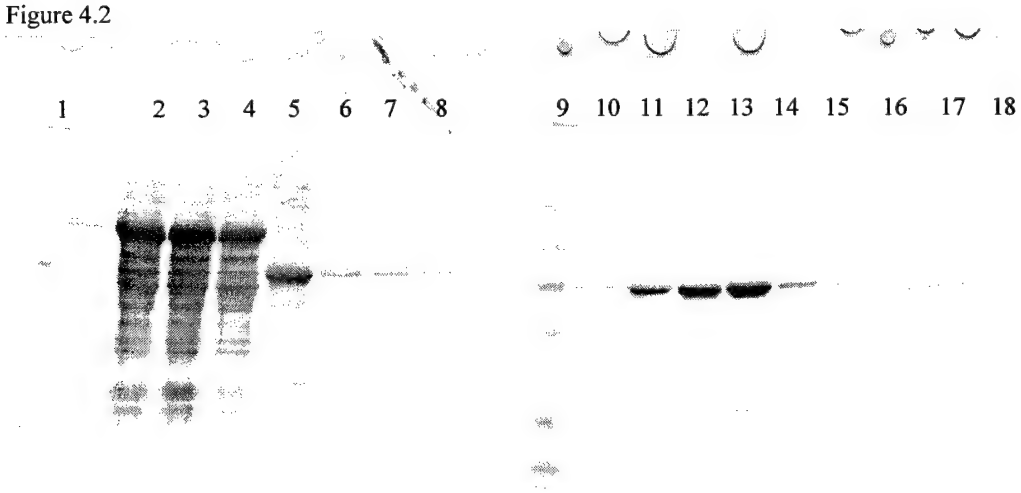
The initial capture step was done with Pharmacia SP-Sepharose FF cation exchange resin in batch mode. The resin was charged with 3-CVs of high ionic strength buffer (1M NaCl, 50mM sodium phosphate, pH 7.4) followed by equilibration with 5-CVs of equilibration buffer (50 mM sodium phosphate, pH 7.4). The diluted, filtered, lysate supernatant was mixed with the equilibrated resin and allowed to mix for three hours with gentle stirring at ambient temperature. Then the resin was poured into a column and packed by pumping the equilibration buffer through the column at 113 cm/hr. The column was washed with 4-CVs of equilibration buffer to remove unbound *Pichia* proteins, then the product was eluted with a step to 200mM NaCl for 4-CVs.

The 200 mM SP-elution pool was diluted 1/3 with equilibration buffer (50 mM sodium phosphate, pH 7.4), then applied to a Pharmacia Q-Sepharose FF anion exchange column at a flow rate of 150cm/hr. The flow-through peak was collected (product does not bind).

The Q-flow-through pool was concentrated 3X and diafiltered against 25 mM succinic acid, pH 5.0 for 6 diafiltration volumes. The product was applied to a Pharmacia Source-15 S cation exchange column at 150 cm/hr. The column was washed with 5-CVs of equilibration buffer (25 mM succinic acid, pH 5.0) at 300 cm/hr. The product was eluted with a linear gradient over 30-CV from 0 to 450 mM NaCl. The BoNTE Hc fragment was collected from the first peak in the linear gradient and stored at -20°C.

The process at the first three steps generated a product that is approximately 95% pure based on SDS-PAGE (Figure 4.2, lanes 11-13). The primary contaminant is a 17 kD proteins that has been seen with other BoNT Hc preparations, in particular BoNTA Hc. To remove this 17 kD protein HIC was investigated.

Figure 4.2

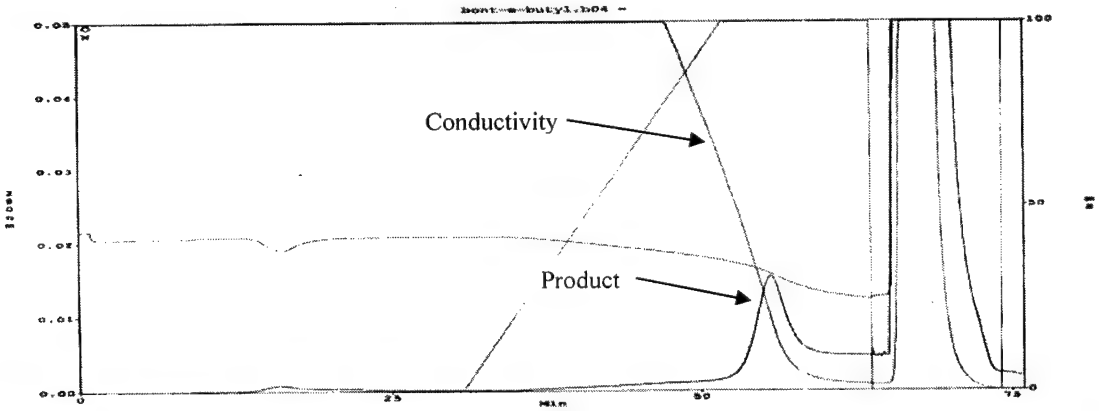


Lane #	Sample	Lane #	Sample
1	MW See Blue	7	Q-Load
2	Lysis Pellet	8	Source 15-S Load
3	Lysis S/N	9	MW See Blue
4	Pre Batch (filtered)	10	Source 15-S Load (dilute)
5	Post Batch (unbound)	11-13	Source 15-S Product fractions
6	Batch Elution Pool	14-18	Later 15-S elution fractions

Hydrophobic interaction chromatography polish step

Several different types of resins and hydrophobicity were evaluated for binding conditions and elution profile. TosoHaas Phenyl 650M and Butyl 650M and Pharmacia Sepharose FF Butyl were tested on the product fraction from Source S-15 column. The columns were equilibrated with 1.5 M $(NH_4)_2SO_4$ in 25 mM NaAc/HAc, pH 5.0 buffer. The BoNTE Hc containing Source S-15 fraction was mixed with $(NH_4)_2SO_4$ to a final concentration of 1.5 M, filtered and then loaded one of the HIC columns, washed with 10 column volume (CV) of the column equilibration buffer. Elution was performed with a 10 CV gradient from 1.5 M to 0 M of $(NH_4)_2SO_4$ in 25 mM NaAc/HAc buffer. All three resins behaved in a similar fashion with the product not completely eluting at the end of the gradient (Figure 4.3). The protein recovery was low in each case with protein eluting in the 1 N NaOH wash step. These results indicate that BoNTE Hc tightly binds to the resin and that an additive will be required to reduce the hydrophobic interaction with the resin.

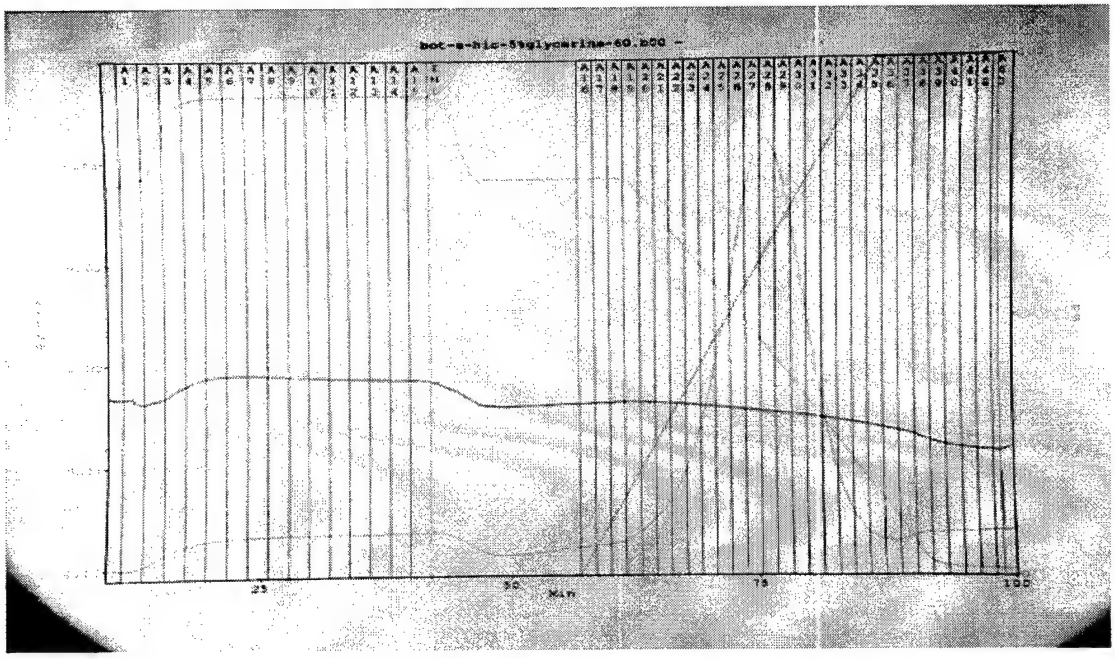
Figure 4.3. Chromatogram of BoNTE Hc on Butyl Sepharose 4FF

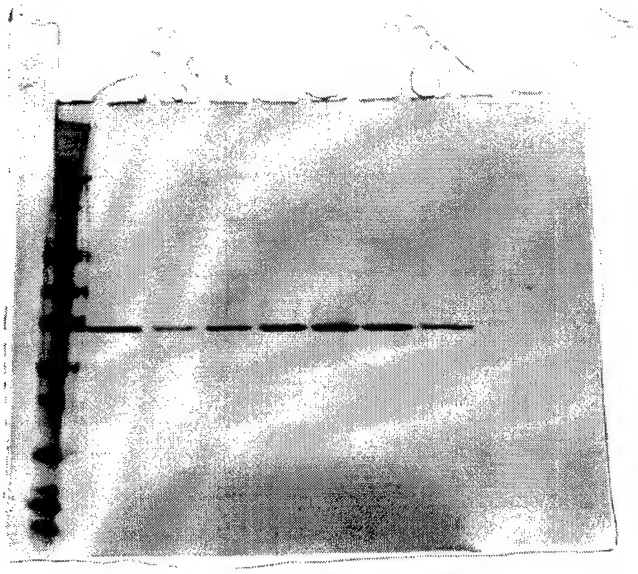


effectively reduce the hydrophobic interaction of BoNTE Hc there is concern of using IPA in a production environment because of the flammable nature of IPA.

As an alternative glycerol was evaluated at 5% by volume in both buffers. The column is a butyl Sepharose 4FF (100 ×Φ10 mm, 7.8ml total volume) equilibrated with Buffer A (0.5 M $(NH_4)_2SO_4$-25 mM NaAc/HAc, pH 5.0 and 5% v/v glycerol). Sixty ml of sample (pooled Source S-15 fraction at a protein concentration of approximately 0.1 mg/ml) containing 0.5 M $(NH_4)_2SO_4$-25 mM NaAc/HAc and 5% v/v glycerol, pH 5.0) was loaded and then washed with 8 CV of Buffer A. The column is eluted with a linear gradient from Buffer A to Buffer B (25 mM NaAc/HAc and 5% v/v glycerol, pH 5.0) over 10 CV (Figure 4.4).

Figure 4.4. Chromatogram and SDS-PAGE of BoNTE Hc on Butyl Sepharose 4FF eluted with 5% glycerol-NaAc buffer system.





Lane 1:Source S-15 pooled
Lane 2: fraction 22
Lane 3: fraction 24
Lane 4: fraction 26
Lane 5: fraction 28
Lane 6: fraction 30
Lane 7: fraction 32

Based on these results the 17 kD protein is removed and is likely in the flow through, which is unconfirmed at this time. This work was completed at the very end of the project and time did not allow for the process to be tested from start to finish. All of the results presented for the HIC step were performed with product pool from the Source S-15 step. The next phase is to test the process from start to finish and to develop a yield table. The product from this HIC study was sent to USAMRIID for animal testing and results indicated that this material protected (Personnel Communication, Dr. L. Smith).

5. BoNTF Hc

Below is a manuscript that has been prepared for submission which describes all of the activity in the BPDF on BoNTF Hc. The BoNT F Hc generated from this work has been tested and protects in mice.

Scale-up of the Fermentation and Purification of the Recombinant Heavy Chain Fragment C of Botulinum Neurotoxin Serotype F, Expressed in *Pichia pastoris*

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Abstract

A recombinant heavy chain fragment C of botulinum neurotoxin serotype F (BoNTF(Hc)) has been expressed in *Pichia pastoris* for use as an antigen in a proposed human vaccine. *P. pastoris* cells were grown using glycerol batch, glycerol fed-batch and methanol fed-batch methods to achieve high cell densities. The total cellular protein recovered after homogenization was 72 mg per gram of cell paste. BoNTF(Hc) was purified from soluble *Pichia* cell lysate using a modified method developed by Covance, Inc. employing ion-exchange chromatographic (IEC) and hydrophobic interaction chromatographic (HIC) methods. This process was performed at the pilot-scale. The modified process resulted in greater than 98% pure product consisting of two forms of BoNTF(Hc) based on SDS-PAGE and yielded up to 205 mg/kg cells at the bench scale and 170 mg/kg cells at the pilot scale based on the BCA protein assay. N-terminal sequencing shows the two forms are –11 (80%) and –14 (20%) from the full-length form. The ratios of these two forms were consistent from the bench to pilot scales.

Introduction

There are seven serologically distinct forms (A-G) of botulinum neurotoxins (BoNT) produced by *Clostridium botulinum*, each of which are the most toxic substances known to man (1). Exposure to these agents results in the disease botulism, which leads to fatal paralysis of respiratory muscles (2). BoNT act on nerve cells by inhibiting the release of the neurotransmitter acetylcholine, at neuromuscular junctions, preventing muscle activation, leading to flaccid paralysis (3-4).

Active BoNT are produced by post-translational modification of a 150 kDa precursor, resulting in a dimer consisting of a 100 kDa heavy chain and a 50 kDa light chain connected by a disulfide bond (5-6). The C-terminus of the heavy chain (Hc) acts to bind to specific receptors on cholinergic nerve cells (7-12). The N-terminal portion of the heavy chain functions in translocation of the light chain of the neurotoxin across the endosomal membrane (13-16). The light chain, a zinc-endopepsidase (4, 17-19), then attacks key synaptic vesicle proteins, which are required for neurotransmitter release (4, 17-20). For nerve intoxication to completely occur, all portions of the BoNT must be present. The Hc fragments have been found non-toxic, antigenic (21-22) and have been shown able to elicit a protective immunity in animals challenged with BoNT (23-24). Therefore, these Hc fragments are being investigated as a method of vaccination in humans for protection against botulinum (24-29).

A fermentation protocol utilizing glycerol batch, glycerol fed-batch and methanol fed-batch phases allowed high cell densities to be reached. The induction time was kept short to optimize BoNTF(Hc) production while minimizing the proteolytic degradation. A purification method developed by Covance, Inc. was tested and some modifications were made, mainly in the harvesting step. Initial runs performed at the bench-scale resulted in much lower product yields than expected, which led us to optimize the cell breakage step. A total of three bench-scale runs were performed using cells generated from either a 60 L or 5 L fermentation and two pilot-scale purification runs were performed using cells generated from the same 60 L fermentation to determine the ability of this method to be scaled-up. Unless otherwise noted, cells were processed immediately after harvesting.

Materials and Methods

5 Liter and 60 Liter Fermentation. A 1-L baffled shake flask containing 150 mL BMGY (buffered minimal glycerol complex medium, *Pichia* Expression Kit, Invitrogen Co., USA) was inoculated with 1 mL of frozen strain stock and incubated at 30 ^{0}C, 200 rpm until an OD_{600} (optical density at 600 nm) between 4-8 was reached. The entire 150 mL was employed as an inoculum for a 5-L fermentor (Bioflo 3000, New Brunswick Scientific Co., USA) containing 3 L of BSM plus 0.435% v/v PTM1. BSM consists of (per L) 26.7 mL 85% H_3PO_4, 0.93 g $CaSO_4$, 18.2 g K_2SO_4, 14.9 g $MgSO_4 \cdot 7H_2O$, 4.13 g KOH, and 40.0 g glycerol; and PTM1 consists of (per L) 6.0 g $CuSO_4 \cdot 5H_2O$, 0.08 g NaI, 3.0 g $MnSO_4 \cdot H_2O$, 0.2 g $Na_2MoO_4 \cdot 2H_2O$, 0.02 g H_3BO_3, 0.5 g $CoCl_2$, 20.0 g $ZnCl_2$, 65.0 g $FeSO_4 \cdot 7H_2O$, 0.2 g biotin and 5.0 mL H_2SO_4). Prior to inoculation, the pH was adjusted to 5.0 with concentrated ammonium hydroxide. The fermentation was controlled at pH 5.0, temperature 30 ^{0}C and dissolved oxygen (D.O.) >20% of saturation. When the fermentation was grown to 30 - 50 g/L wet cell weight (WCW), the culture was transferred to a 80-L fermentor (MPP 80, New Brunswick Scientific Co.) containing 40 L of BSM plus 0.435% PTM1. When glycerol was exhausted, which was indicated by a D.O. spike, a glycerol feed was initiated and lasted for 4 hours. The feed rate has the following profile: 1st h, 20 g/h/L (g 50% w/v glycerol containing 1.2% v/v PTM1 per h per L initial medium); 2nd - 4th h, feed rate decreased linearly from 20 g/h/l to 0. At the end of 1st h, 1.5 g of methanol was added to the fermentor. At the end of 3rd h, a methanol feed was initiated and the feed rate was programmed to increase linearly using the following profile: 0 h, 4 g/h/L (g 100% methanol containing 1.2% v/v PTM1 per h per L initial medium); 2.4 h, 6 g/h/L; 3.8 h, 7 g/h/L; 8.5 h, 9 g/h/L. The methanol feed rate was adjusted using the dissolved oxygen spike method (30). Cell mass was harvested at 8.5 h. The total induction time on methanol was 10.5 h. The final cell density was about 170 g/L wet weight.

5 Liter Cell Harvest and Disruption: Cells harvested from a 5 L fermentation were spun at 8000 x g at 4 °C for 20 min using a Beckman J2-21 Centrifuge (Palo Alto, CA). Cell paste was then either frozen at –20 °C or processed immediately. Cell paste was resuspended in 25 mM sodium acetate + 5 mM EDTA, pH 5.0 to either 10 or 26% (w/v) solids. Cells were then homogenized using a Microfluidizer M-110EH (Microfluidics, Corp., Newton, CA) set at 21,000 psi. A total of 3 to 5 passes were performed to obtain at least 75% cell disruption. The homogenate was then brought to 0.25% Polyethyleneimine (PEI) using a

5% (w/v) stock solution at pH 7.0. This was allowed to mix for 30 min at 4 °C. The resulting mixture was processed by centrifugation at 10,000 x g for 20 min at 4 °C. The supernatant was then decanted, passed through a 0.2 micron CA filter and saved for further immediate processing.

Bench-scale Purification. All chromatographic separations were performed on a BioCad Workstation (PE Biosystems, Foster City, CA) at room temperature, with the load material on ice. Filtered supernatant was loaded onto a 100 mL SP Sepharose FF column (2.6 x 19 cm) (Amersham Pharmacia) equilibrated with 5 column volumes (CV) of 25 mM sodium acetate + 1 mM EDTA, pH 5.0, at a linear velocity of 250 cm/h. The columns was then washed with 5 CV equilibration buffer, followed by a wash step with 25 mM sodium phosphate + 1 mM EDTA, pH 6.8. The product was then eluted from the column using a 20 CV linear gradient from 0 to 1 M sodium chloride in 25 mM sodium phosphate + 1 mM EDTA, pH 6.8. The product begins eluting at about 20 mS conductivity.

The SP Sepharose product was then brought to 1 M ammonium sulfate using a 3.5 M ammonium sulfate stock. The resulting solution was filtered through a 0.2 micron filter. The filtered material was then loaded onto a 32 mL Butyl Sepharose 4 FF column (1.6 x 16 cm) (Amersham Pharmacia) equilibrated with 5 CV 1 M ammonium sulfate + 25 mM sodium phosphate + 1 mM EDTA, pH 6.8, at a linear velocity of 300 cm/h. The column was washed with 5 CV of equilibration buffer and the product was eluted using a 10 CV linear gradient from 1 to 0 M ammonium sulfate in sodium phosphate + 1 mM EDTA, pH 6.8. BoNTF(Hc) elutes as the second major peak at 60 mS conductivity (Figure ??).

The Butyl Sepharose product was then dialyzed vs. 20 volumes of 25 mM sodium acetate + 1 mM EDTA, pH 5.0 using 10 kDa Slide-A-Lyzer cassettes (Pierce, Rockford, Il) at 4 °C. The dialyzed product was then loaded onto a 13 mL ToyoPearl SP 650M column (1.0 x 17 cm) (TosoHaas, Montgomeryville, PA) equilibrated with 5 CV of 25 mM sodium acetate + 1 mM EDTA, pH 5.0, at 300 cm/h. After loading, the column was washed with 5 CV of equilibration buffer and BoNTF(Hc) eluted as the second major peak (13 mS conductivity) near the end of a 20 CV linear gradient from equilibration buffer to 100 mM sodium phosphate + 1 mM EDTA, pH 7.0.

60 L Cell Harvest and Disruption. Cells were harvested using a Westfalia CSA8 disk-stack separator (Oelde, Germany). Fermentation broth was diluted to 10% solids with distilled water. The resulting cell paste (50 to 60% wet solids) was either frozen at –20 °C or processed immediately. The cell paste was

brought to 21-26% (w/v) solids using 25 mM sodium acetate + 5 mM EDTA, pH 5.0 and disrupted using an APV Gaulin 30-CD Homogenizer at 16000 (Everett, MA) psi at 10 °C. Cells were exposed to 3 to 5 passes through the homogenizer and cooled to 10 °C after each pass. The homogenate was brought to 0.25% PEI using a 5% (w/v) stock and allowed to mix for 30 min at 10 °C. The solids were removed by centrifugation using the CSA8 Westfalia Centrifuge at a flow rate of 2 L/min. The supernatant was collected for immediate processing.

Pilot-scale Purification. All chromatographic separations were performed on a NC-SRT Pilot-scale Chromatography skid. For each chromatographic step, conditions were the same as the bench-scale work, ie. same linear velocity, equilibration, washing and elution methods. The SP Sepharose FF step was performed using a 4 L (20 x 13 cm) BPG 200/500 column (Amersham Pharmacia Biotech, Piscataway, NJ). The Butyl Sepharose 4 FF step was performed on a 1.3 L (10 x 16 cm) BPG 100/500 column (Amersham Pharmacia), while the final ToyoPearl SP 650M step was performed using a 526 mL (6.1 x 18 cm) Vantage 60A column (Millipore, Bedford, MA). The SP Sepharose product was brought to 1 M ammonium sulfate by addition of granular ammonium sulfate. The diafiltration step was performed using a 3 sq. ft. 10 kDa spiral wound membrane (Millipore). The material was diafiltered with 3 volumes of 25 mM sodium acetate + 1 mM EDTA, pH 5.0, at which time the retentate pH was 5.0 and the conductivity was 4.7 mS at 10 °C. The retentate was then concentrated about 2-fold and filtered through a 0.45 micron CA filter prior to loading onto the final column.

Protein Analysis Total protein concentrations were determined using the BCA (Pierce) Standard Assay, using BSA to generate the standard curve. Purity was determined by SDS-PAGE using 4-20% polyacrylamide gels (Novex, San Diego, CA) stained with coomassie blue or silver stain. Western blot analysis was performed using polyclonal Protein G-Sepharose-purified horse anti-BoNTF antibody incubated at 1 μg/mL for 1 h. The secondary antibody was a horseradish peroxidase labeled affinity-purified goat anti-horse IgG (Kirkegaard & Perry Laboratories, Gainsburg, MD) incubated at 1 μg/mL for 1 h. SDS-PAGE-separated proteins were transferred to PVDF membranes (BioRad, Hercules, CA) at 100 V for 1 h, blocked with 5% non-fat dry milk for 1 h and washed with 0.05% Tween-20 in phosphate-buffered saline (PBS) prior to treatment with antibodies. Blots were then visualized by Chemiluminescence using the ELC plus Detection Kit (Amersham Pharmacia Biotech, Piscataway, NJ). N-terminal sequencing was

performed by the University of Nebraska-Medical Center Protein Core Facility using automated Edman degradation performed on a Procise model 491-HT amino acid sequencer (PE Biosystems, Foster City, CA).

Results and Discussion

Bench-scale Fermentation and Purification Fermentations performed at the 5 liter scale involved standard methanol feed rates, with up to a 10 h induction. This short induction time was performed to minimize the effect of proteolytic cleavage in the fermentation, yet still reach sufficient levels of expression. An initial run was performed following the process described by Covance Inc., which resulted in a significantly lower product yield than expected (54.35 mg BoNTF(Hc)/kg cell) compared to published results (24). A review of the method suggested that breakage at a higher % solids would result in a much higher amount of cell lysis. An experiment was performed to test the amount of total protein released by homogenization at different % solids (see figure 1). These results show a 6-fold increase in the amount of protein released by increasing the % solids from 10 to 26%. We also found that for optimum release of protein during disruption, harvested cell paste should be processed immediately and not frozen. It is believed that the freezing of cells causes them to become rigid and more difficult to disrupt (28). Therefore, subsequent cell disruptions were performed at 26% solids and were conducted immediately after harvesting.

A total of three purification processes were performed following the described method above, to determine reproducibility. Results of these purification runs were very similar in product yield, purity and elution profiles. The entire purification process, from harvesting to final sterile-filtering, was performed in 20 h. Purification runs resulted in final product yields, ranging from 155 mg to 205 mg BoNTF(Hc)/kg cells (see table 1). The maximum amount of total protein loaded onto the SP Sepharose FF column was 55.5 mg/mL resin. At this load less than 3% of the product was seen in the flow through. A maximum load of 4.8 mg/mL was used for the Butyl Sepharose 4 FF column and 2.7 mg/mL for the final ToyoPearl SP 650M column. No BoNTF(Hc) was detected in flow through samples of the HIC or final SP columns.

A typical purification resulted in at least 98% pure BoNTF(Hc) which consisted of two forms of the product (see figure 2). Analysis by N-terminal sequencing shows the prominent form (80% of the total) of the product to be missing the first 11 amino acids (a.a.) and a second form missing the first 14 a.a. of the

amino terminus (see table 2). This ratio of product forms was consistent for all purification processes performed at both the bench and pilot scales. SDS-PAGE analysis of in-process samples show the two product forms to exist in the lysate, suggesting they may be the result of proteolytic degradation in the fermentor. A fermentation was performed with a shortened methanol induction time of 3 h, in an attempt to produce sufficient amounts of full-length BoNTF(Hc). Immediate purification of this fermentation resulted in a 2-fold reduction of product yield and N-terminal sequence analysis shows two forms of the product, similar to previous purifications.

An attempt to remove the minor high molecular weight contaminants present in the BoNTF(Hc) final product was performed using the zwitterionic detergent, CHAPS as an additive in the initial column buffers. We reasoned that these contaminants may be involved in a protein-protein interaction with the product and therefore are not being separated during the multi-chromatographic process. We found that 0.25% CHAPS did not aid in the removal of these proteins (data not shown). Also unsuccessful was an attempt using a negative purification step with Q Sepharose FF following the capture step with CHAPS present in the buffers. BoNTF(Hc) from this process did not show an increase in purity. These contaminants were removed however, by making a very detailed cut in collection of product eluting from the SP Sepharose FF column. Such detail in fraction collecting is difficult to perform at the pilot scale, and leads to lower product yields as the elution of these contaminants and BoNTF(Hc) overlap.

Pilot Scale Fermentation and Purification All work performed in the pilot plants employed use of batch records for process transfer to a GMP manufacturing plant. A total of two 60 liter fermentations and two purification runs were performed. Pilot scale fermentations resulted in final wet cell weights ranging from 21.2% to 30.8% with final volumes of 56 and 57 L.

A total of two purification runs were performed at the pilot scale. The initial run was performed immediately after harvesting of cells, and resulted in 179 mg BoNTF(Hc)/kg cell (see Table 2). The product from the SP Sepharose FF capture column was collected in bulk with collection beginning as soon as the Abs_{280} began to increase. Upon completion of this purification run, the final product was found to contain two minor higher molecular weight contaminants (data not shown). Further testing of the method showed these contaminants were the result of the type of cut made during product collection off the capture column. These contaminants co-elute significantly with BoNTF(Hc) and proved difficult to remove during

subsequent chromatography steps (see above). A second pilot run was performed using frozen cells from the same fermentation run and resulted in a highly pure product (see figure 3), however, showed five-fold less BoNTF(Hc) yield. To avoid collection of the higher molecular weight contaminants, product collection began only at the apex of the elution peak. This would account for approximately half of the product typically collected. The remaining product loss occurred during recovery of soluble material after treatment with PEI (data not shown). Without an HPLC or ELISA method for detection of BoNTF(Hc) it is difficult to determine the actual product loss, however these estimates were generated based on total protein data.

The entire purification process from harvesting to final sterile-filtering took about 25 h. This is similar to the time required for small scale processing. Overall processing at the pilot scale was smooth and resulted in a similar product yield per kg cell. The changes in processing relative to the bench scale were in the type of equipment used to perform certain tasks, ie. laboratory centrifuge vs. disk-stacked separator or dialysis cassettes vs. diafiltration systems. In order to remove cell debris and flocculated nucleic acid from cellular homogenate, a disk-stacked separator was used rather than conventional laboratory centrifugation. Use of this equipment can result in a similar product yield obtained in the laboratory (see table 1 & 3). At the bench scale this step took 30 min, while at the pilot scale 1 h was required. Of the total protein released during homogenization of the initial pilot run, approximately 44% remained in the PEI-treated supernatant, however processing during a second run recovered only 9% of the total protein released.

To adjust the Butyl Sepharose 4 FF product to 25 mM sodium acetate + 1 mM EDTA, pH 4.5, a diafiltration system was used with a jacketed stainless-steel sample reservoir, chilled to 7 °C. This step resulted in an insignificant loss of product and took only 2 h to perform using a 10 kDa spiral-wound regenerated cellulose membrane. During adjustment of the SP Sepharose FF product to 1.0 M ammonium sulfate, some precipitation was seen initially, however if added slowly in increments over a 1 h period with continuous mixing, this was minimized. This phenomenon was more prominent at the pilot scale, possibly due to an increased sample temperature upon mixing. During adjustment at the pilot scale, the sample was approximately 17 °C, three times the temperature this step was performed at for the bench scale process, which may have caused the protein to be less stable.

Conclusion

The production and purification of recombinant BoNTF(Hc) from *Pichia pastoris* using the process described above has been scaled-up and can result in a similar product yield and purity at both the bench and pilot scales. Fermentations achieved high cell densities, and reached optimum BoNTF(Hc) production after 10 h induction. It was found that to achieve maximum BoNTF(Hc) yield, purification should be performed immediately upon completion of the fermentation. The purification process involves three chromatographic steps, which is typical of methods used to purify non-secreted products from *Pichia*, and results in a consistent ratio of two nicked forms of pure BoNTF(Hc).

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Figure Captions

Figure 1. Effect of % Solids on Cell Breakage. Cells harvested from 5 L and 60 L fermentations were homogenized and exposed to centrifugation. Resulting supernatants were analyzed for total protein concentration using the Bradford protein assay (BioRad) using BSA as the standard.

Figure 2. (A) Silver stained SDS-PAGE and (B) Chemiluminescent western blot of purified BoNTF(Hc) from a typical bench-scale purification (PDL-004). (A) Lane 1 is MW standards, from top, 250 kDa, 98 kDa, 64 kDa, 50 kDa, 36 kDa, 30 kDa, 16 kDa, 6 kDa, 4 kDa, Lane 2 is μg BoNTF(Hc) std (Covance, Inc), Lane 3 is 5 μg final product , Lane 4 is 2.5 μg final product, Lane 5 is 1 μg final product. (B) Lane 1 is 200 ng final product, Lane 2 is 50 ng final product, Lane 3 is 30 ng final product.

Figure 3. (A) Coomassie stained SDS-PAGE and (B) Chemiluminescent western blot of purified BoNTF(Hc) from a pilot-scale purification (PPP-007). (A) Lane 1 is 5 μg final product, Lane 2 is 4 μg final product, Lane 3 is 3 μg final product, Lane 4 is 2 μg final product, Lane 5 is 1 μg final product, Lane 6 is MW standards, from top, 250 kDa, 98 kDa, 64 kDa, 50 kDa, 36 kDa, 30 kDa, 16 kDa, 6 kDa, 4 kDa, Lane 7 is 3μg BoNTF(Hc) std (Covance, Inc.). (B) Lane 1 is 70 ng final product, Lane 2 is 50 ng final product, Lane 3 is 40 ng final product, Lane 4 is 20 ng final product, Lane 5 is 10 ng final product, Lane 6 is blank, Lane 7 is 15 ng BoNTF(Hc) std.

List of Abbreviations

BCA	bicinchoninic acid
BoNT	botulinum neurotoxin
BoNTF(Hc)	botulinum neurotoxin serotype F, heavy chain C-terminal fragment
BMGY	buffered minimal glycerol complex medium
BSA	bovine serum albumin
BSM	basal salts media
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CV	column volume
DO	dissolved oxygen
EDTA	ethylenediaminetetraacetic acid, disodium
ELISA	enzyme-linked immunosorbent assay
FF	fast flow
GMP	good manufacturing practice
Hc	heavy chain C-terminal fragment
HIC	hydrophobic interaction chromatography
HPLC	high performance liquid chromatography
IEC	ion-exchange chromatography
KDa	kilodalton
MW	molecular weight
PEI	polyethyleneimine
PTM1	*Pichia* trace minerals 1
PVDF	polyvinylidene difluoride
Q	quaternary amine
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SP	sulfopropyl
WCW	wet cell weight

Table 1. Bench Scale Purification of BoNTF(Hc)[a]

Step	Volume (mL)	$[Protein]_{Total}$ (mg/mL)	Total Protein (mg)	Purity[b] (%)	BoNTF(Hc)[c] (mg)	Fold Purification
Lysate	820	13.26	10873	<0.5	54.4	1
PEI-treatment	740	7.65	5661	<1	45.3	1.6
SP Sepharose FF	270	0.64	174	25	43.4	50
Adjusted SP Seph FF	380	0.42	161	26	41.9	52
Butyl Sepharose 4 FF	87	N.D.	N.D.	87	N.D.	N.D.
Dialyzed Butyl Seph 4 FF	88	0.43	37.9	87	33.0	174
ToyoPearl SP 650M	65	0.36	23.3	98	22.9	196

[a]Purification is from 150 g cells wet weight.

[b]Estimated by visual inspection of SDS-PAGE.

[c]Determined by multiplication of total protein times purity.

N.D. Not determined

Table 2. N-terminal Sequence of Purified BoNTF(Hc)

Sample	N-Terminal Sequence
Intact BoNTF(Hc)	**MSYTN DKILI LYFNK LYKKI KDNSI**
Bench scale PDL-004	**YFNK LYKKI K 80% (-11)** **K LYKKI XDNX 20% (-14)**
PDL-005	**YFNK LYKKI K 75% (-11)** **K LYKKI KDN 25% (-14)**
PDL-006	**YFNK LYKKI K 76% (-11)** **K LYKKI XDNX 24% (-14)**
PDL-007	**YFNK LYKKI K 80% (-11)** **K LYKKI KDN 20% (-14)**
Pilot scale PPP-006	**YFNK LYKKI K 89% (-11)** **K LYKKI KDN 11% (-14)**
PPP-007	**YFNK LYKKI K 77% (-11)** **K LYXXI XDNS 23% (-14)**

Table 3. Pilot Scale Purification of BoNTF(Hc)[a]

Step	Volume (L)	$[Protein]_{Total}$ (mg/mL)	Total Protein (g)	Purity[b] (%)	BoNTF(Hc)[c] (mg)	Fold Purification
Lysate	23	13.72	316	<2	4.7	1
PEI-Treatment	90	1.56	140	<3	3.5	1.7
SP Sepharose FF	20	0.69	13.8	25	3.5	17
Adjusted SP Seph FF	29	0.24	7.0	33	2.3	22
Buytl Sepharose 4 FF[d]	5.6	0.41	2.3	80	1.9	56
Diafilterd Butyl Seph 4 FF	5	0.50	2.5	80	1.9	54
ToyoPearl 650M	4.1	0.26	1.1	98	1.0	61

[a]Purification is from 5.95 kg cells wet weight.

[b]Estimated by visual inspection of SDS-PAGE.

[c]Determined by multiplication of total protein times purity.
[d]Precipatation occurred during Amm. Sulfate addition.

Figure 5.1

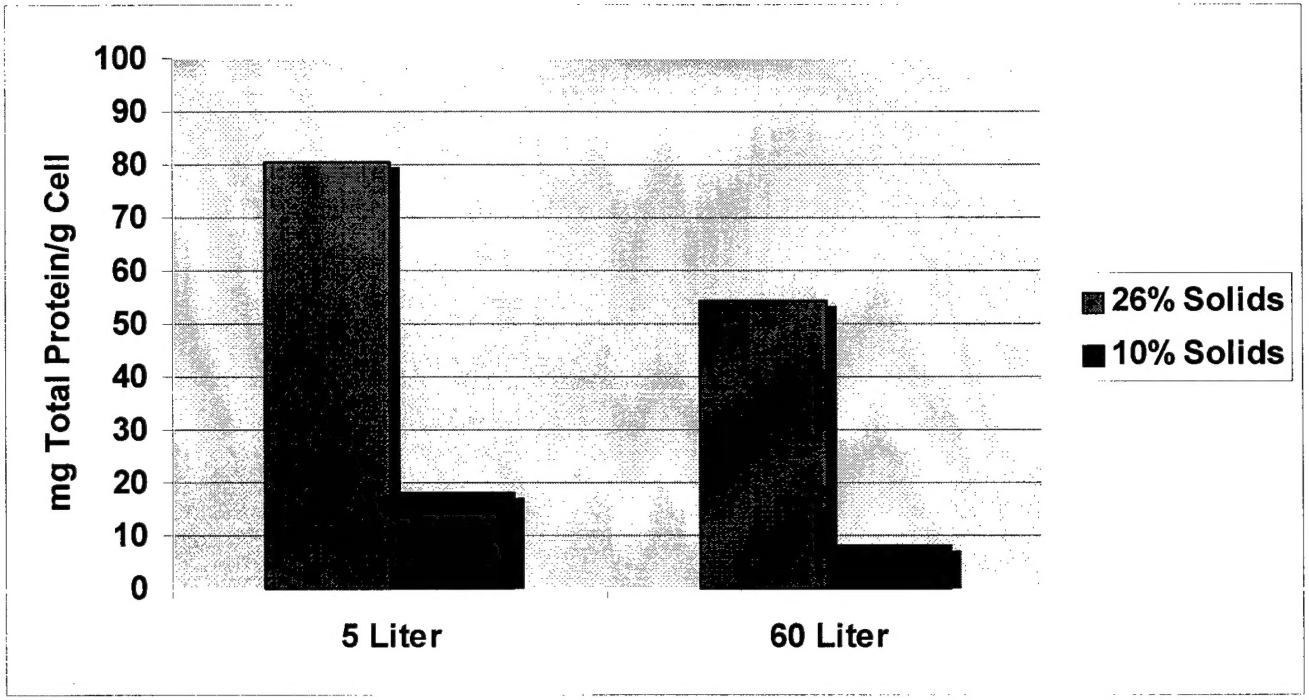
mg Total Protein/g Cell
100
90
80
70
60
50
40
30
20
10
0
5 Liter
60 Liter
26% Solids
10% Solids

Figure 5.2

SDS-PAGE **WESTERN**

1 2 3 4 5 **1 2 3**

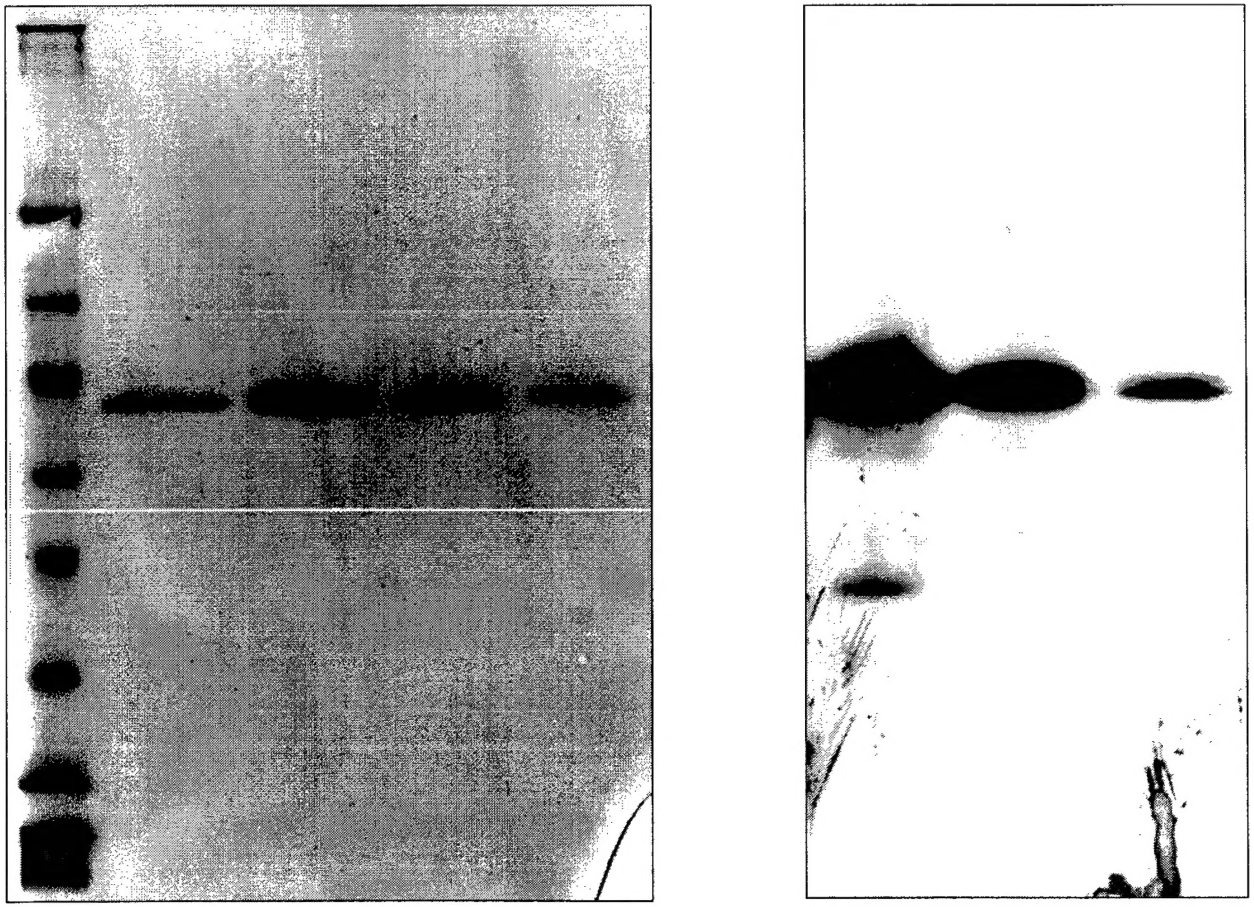

Figure 5.3